Cover Page

STUDY TITLE:

Abnormal Food Timing and Circadian Dyssynchrony in Alcohol Induced Colon Carcinogenesis

Short title:

AFT

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11. RESEARCH STRATEGY

A. SIGNIFICANCE

Alcohol intake, even in small amounts, increases the risk of colonic pre-malignant lesions (polyps) and colorectal cancers (CRC)[1, 2]. But only a subset of drinkers develops CRC. This could be due to co-factors that modify the CRC risk from alcohol use[3]. Our knowledge of such factors and mechanisms that potentiate CRC development in alcohol drinkers is limited. The goal of our proposed studies is to fill these knowledge gaps.

Alcohol, Eating time, and CRC: Factors associated with modern lifestyles are linked with the high incidence of CRC in developed countries, while less-modern populations have a lower risk of CRC[4]. Besides alcohol, the Western diet is associated with an increased CRC risk[5]. The uptrend in CRC rates is being observed in populations embracing more modern lifestyle[4, 6]. With recognition of the role of circadian disruption in pathogenesis of chronic diseases[7], new studies provide compelling evidence that changes in the timing of food consumption, especially "a delayed eating pattern" (e.g., night time eating) disrupts intestinal circadian rhythms [8-10], promotes a pro-inflammatory state that is associated with metabolic syndrome[11-16], a condition associated with colon polyps and cancers[17-19]. Indeed, shift-workers, who also have disrupted circadian rhythms, are at increased risk of CRC [20], which may be a consequence of central (light:dark cycles) or peripheral (time of eating) circadian rhythm disruption. About 20% of our genome is under circadian control and disruption of circadian rhythms can disturb tissue homeostasis, increasing susceptibility to pathologies including cancer [21, 22]. While light:dark cycles are the main regulator of our central clock, located in the brain [in the SCN], food is the main regulator of the circadian rhythms in the digestive tract[16, 23]. Delayed eating (i.e., eating larger meals during biological rest time), a frequently observed phenomenon in our modern society [24, 25], can cause a mismatch [dyssynchrony] between intestinal and central circadian rhythms, leading to pathology [26]. Prior animal studies indicate that feeding during rest time shifts the colonic circadian rhythm, and desynchronizes it from the central clock [8, 9] This circadian dyssynchrony may facilitate carcinogenesis upon repeated exposures to alcohol. Although the factor of (eating) time has been overlooked in the prior epidemiologic studies, the link between alcohol, food timing and CRC appears to be scientifically logical and is supported by our data in the mouse model of polyp/CRC.

Scientific premise: Emerging evidence strongly suggest that low-grade inflammation could be the common pathway through which environmental factors associated with modern lifestyle increase the risk of CRC. For example, pro-inflammatory state in metabolic syndrome and obesity, common conditions in western societies increase CRC risk [18, 27-30]. Alcohol, another CRC risk factor induces inflammation in the gut characterized by oxidative stress, alterations in innate and adaptive immunity, and augmented release of pro-inflammatory cytokines [31-35]. Our group showed that alcohol-induced gut inflammation is associated with accelerated polyposis in a mouse model of polyposis, supporting inflammation as a mechanism underling alcohol-induced carcinogenesis [36]. Studies have established that intestinal inflammation facilitates a microenvironment that promotes cell proliferation, adhesion, and invasion [37, 38]. Yet, not all alcoholics develop CRC supporting the hypothesis that another factor that could also promote intestinal inflammation is required to increase CRC risk. Disrupted circadian rhythms and homeostasis could be such a candidate because (1) it promotes pro-inflammatory state in the colon[39] and causes dysregulated immunosurveillance. Indeed, immune system is under circadian control and circadian desynchronization can lead to chronic inflammation, and multiple related pathologies including cancer [40]; (2) disrupted circadian is also a common feature of modern societies[41] where the CRC risk of CRC is higher. Indeed, shift workers have higher risk of CRC [20].

Our group has shown that central circadian rhythm disruption (of light:dark cycles) enhances mucosal inflammation in animal model of chemically induced-colitis [42]. Also, disruption of central circadian rhythmicity induced by changing light:dark cycles combined with alcohol disrupted intestinal barrier integrity and promoted gut leakiness to endotoxins, a situation that promotes mucosal and systemic inflammation [43]. My novel and exciting preliminary data in mice show that disruption of intestinal circadian homeostasis by an abnormal eating pattern ["wrong time eating"] accelerates the development of alcohol-induced colon cancer [Fig 1]. Further, the abnormal eating pattern shifted the colonic circadian rhythm [Fig 2]. Accordingly, the *objectives* of my K23 application are (**i**) to test the *hypothesis* that *a delayed eating pattern is a key cofactor that increases susceptibility to alcohol-induced colon carcinogenesis* and (**ii**) to elucidate the mechanism. This is *significant* as the first step to better understand how disruption of intestinal circadian rhythms by delayed eating patterns promote alcohol-associated colonic neoplasia. This will have immediate impact in risk

stratification of alcohol consumers for CRC screening, and can be readily extended to the general population in tailoring primary and secondary prevention strategies. Outcomes from these studies will lead to in-depth mechanistic studies and ultimately the discovery of novel targets to prevent CRC. Also, this study will have implications in a broader range of alcohol-related pathologies that are the consequence of gut inflammation.

B. INNOVATION

In this era of personalized/precision medicine, it is prudent to identify host factors that promote alcohol-induced colon carcinogenesis. This project will establish an **innovative discovery**: that delayed eating patterns ("wrong time eating") is a risk factor for alcohol-induced colon carcinogenesis pathways in human. Our prelim data support the promoting role of wrong time eating in alcohol-induced polyposis [Fig 1]. Our **hypothesis –**intestinal circadian dyssynchrony due to delayed eating results in pro-tumorigenic inflammatory profile, associated with enhanced carcinogenesis in susceptible host – is novel. Our translational **experimental approach is innovative.** We will use a combination of animal and human studies – In subjects with history of advanced sigmoid/descending colon polyps, we will assess :(i) Central circadian rhythms by hourly salivary melatonin; (ii) and "Digestive tract" circadian rhythm by 2 hourly buccal and 12 hourly rectal expression of clock genes. This will provide valuable information regarding the impact of eating patterns and alcohol consumption on central and peripheral circadian rhythms and the relationship of these inter-related, yet independent circadian biosystems. Our human study will use a crossover, 2 by 2, design to examine the same individual under 4 conditions (± delayed eating pattern & ± alcohol). Using a crossover design will eliminate inherent inter-individual variations in alcohol metabolism, circadian rhythm, immune system, and microbiota composition. This design will allow for use of sampler sample size and for a more accurate examination of the impact of alcohol and eating conditions on the circadian system and its interaction with mucosal immune profiles, microbiota and carcinogenesis markers. Our **data analysis approach is state of the art and innovative** as we will use systems biology and network analysis specifically transcriptional profiling to assess the effects of alcohol and eating patterns on our outcomes (immune profiles, circadian synchrony, microbiota data, and carcinogenesis markers), and to characterize profiles associated with each experimental condition. We will first establish an **innovative pro-tumorigenic immune mucosal profile** based on RNA-based signatures of our established animal model. We will identify transcriptional profiles in the colonic mucosa that include immune response genes and which are specifically expressed upon occurrence of polyp/cancers. We will use this RNA seq profile and integrate these signatures with other measures collected in this project to assess the impact of food intake patterns on alcohol-associated changes in endoscopic colon mucosal samples in patients, particularly in immune response genes [Aim 3] [Fig. 3]. This **innovative multidisciplinary approach** is possible due to the combined efforts of the five mentors who are well-established investigators in complementary areas of research with previously established collaborative research efforts among them. The mentors were chosen based on their areas of expertise: Keshavarzian lab [my primary mentor and chair of my mentorship committee], direct molecular and cellular mechanisms that underlie alcohol-induced and circadian rhythm disruption effects on the intestine; Burgess lab, measurements of circadian rhythms in humans; Khazaie lab, mucosal inflammation in polyposis and cancer; Green lab, RNA-sequencing; Sékaly lab, systems biology. The goal of this K23 application is to take an integrated approach with mentors from different fields. Their combined expertise will guarantee a successful completion of the proposed studies, and, will provide a unique environment for training the applicant as an independent translational physician-scientist in the field of chrono-oncology and alcohol .

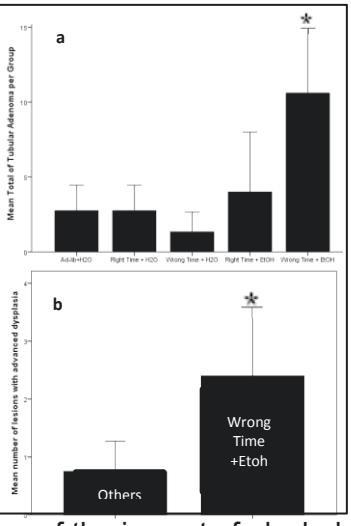

Fig 1. a) Eating at light [rest time/wrong time] vs. eating in dark [active phase/right time] or ad-lib, accelerated alcohol-induced polyposis (far right column) in TS4Cre × cAPC ^{lox468} model. No polyp was seen in wild type B6 animals (data now shown) (n=5 per group). **b)** Aggressive lesions [by severe high grade dysplasia or carcinoma in situ] were more common in "Wrong Time + Etoh" [Right column] animals vs. other groups combined [left column] (*: p<0.01).

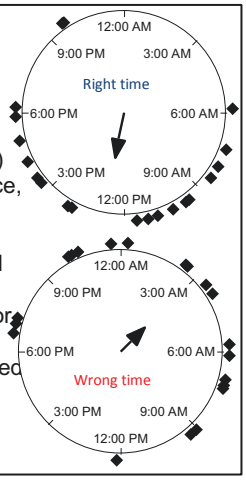

Fig 2. Age-matched B6 PER2::LUC mice were fed during active, "right time", (n=5) or rest phase, "wrong time"/ "abnormal eating" (n=5) for 7 days. After sacrifice, PER2::LUC signaling from the colon tissue sections was monitored for bioluminescence activity every 4 hours for 7 days. Wrong time eating shifted the phase of the colon circadian rhythm (p<0.01).

C. APPROACH.

The *overall goal* of this project is to determine the role of delayed food consumption in promoting alcohol-induced *colon carcinogenesis and begin to elucidate the mechanisms that lead to polyp formation and cancer*. Our **working hypothesis** is that intestinal circadian dyssynchrony induced by delayed eating, enhances alcohol-induced intestinal inflammation, and markers of colonic neoplastic process including epithelial proliferation in susceptible host. We will also investigate the effects of alcohol and food timing changes on colonic microbial composition, as a potential mediator of the predicted outcomes (mucosal inflammation and carcinogenesis). To test this hypothesis, we propose three Aims. In **Aim 1**, we will identify RNA seq signatures that represent field defect in colonic mucosa of susceptible host (APC mice), that promote carcinogenesis. To this end, we will use both normal appearing mucosa and polyps/cancers already collected from colon of alcohol fed ± wrong-time eating TS4Cre×cAPC^{lox468} mice as well as from wild type mice [Fig 3], to identify the pro- and anti-tumorigenic adaptive and innate immune profile induced by alcohol ± wrong time eating, using Immunofluorescence (IF) and RNA-Seq. Using the information from Aim 1, we will use RNA-Seq in human sigmoid tissues in Aim 3 to interrogate the mechanism by which disrupted circadian and alcohol promote carcinogenesis in susceptible subjects (those with history of advanced adenoma). We cannot test polyp samples in humans [see below] and therefore information from our animal study is crucial in determining the RNA seq signature of a "field" predictive of polyp in colon of at risk subjects. In **Aim 2**, we will test the hypothesis that circadian dyssynchrony induced by delayed eating is required to promote carcinogenesis after alcohol consumption in susceptible individuals. In **Aim 3,** we will test the hypothesis that the delayed eating and alcohol, in these susceptible subjects, promotes abnormal mucosal inflammation and proliferation, and that alcohol induced dysbiosis may enhance this effect. We will use a systems biology/network analysis approach to identify interactions between (i) inflammatory profiles (e.g., Treg/Th17) and microbiota composition with (ii) that of markers of CRC in alcohol drinkers with delayed eating patterns.

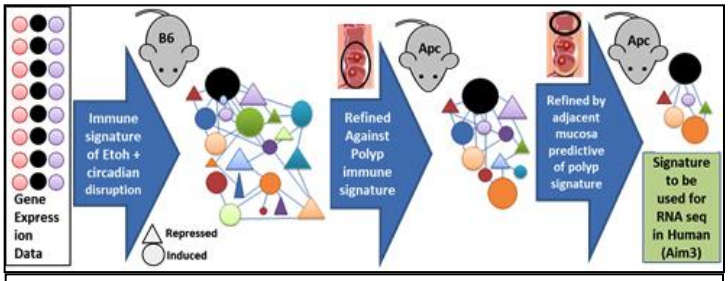

Fig 3: The RNA seq data from colon mucosa of B6 animals will be used to identify the immune Th17/Treg/Neutrophil profile associated with alcohol, wrong time eating or both. This profile will be refined by looking at polyp-based RNA-seq data in APC mice, to subside the immune signature to pathways predictive of colon carcinogenesis. In the next step, cancer predictive profile in the adjacent mucosa predictive of polyp profile will be determined. This profile will be used for Aim3.

Immune candidates. Despite the known link between alcohol and CRC, there is no risk stratification strategy for CRC prevention in alcohol drinkers, likely because the co-factors and mechanisms underlying alcohol-induced CRC are not well understood. The colonic mucosa requires controlled inflammation for homeostasis and defense against microbial pathogens; however, chronic uncontrolled inflammation promotes colon carcinogenesis through changes in mucosal immunity [37, 38]. Components of both the innate and adaptive immune systems are detected in CRC [37]. The immune profile of CRC, in both human and animals, is characterized by a pro-inflammatory state and of higher frequencies of Th17 and concomitant decrease in numbers of regulatory T cells (Tregs) [44-47]. Th17 cells are pro-inflammatory immune cells that interact with microbial antigens in the intestinal lamina propria. Cytokines produced by Th17 cells appear to exert pro-tumorigenic effects, while the presence of a Th17 immune cell infiltrate negatively influences the prognosis of CRC patients [45]. Th17 are regulated and suppressed by Tregs [48]. Tregs are potent inhibitors of inflammation, and could be protective in polyposis and CRC. Accordingly, high intra-tumor densities of Tregs have been correlated with longer survival of CRC patients [49, 50]. **It should also be noted that the abnormal Th17/Treg balance has also been noted in colonic mucosa outside of the polyp/tumors** [51-54]**.** Thus, the balance of Th17/Treg signaling in the adaptive immunity is important for maintaining intestinal homeostasis, which, if disrupted, leads to chronic inflammation and increased cancer susceptibility [55-57]. Neutrophils, a cell subset that plays a major role in the innate immune response, are also involved in immune surveillance, and tumorigenesis in the colon[58, 59], and their abundance relative to adaptive lymphocytes is prognostic in CRC[60]. Both lymphocytes (Th17 and Tregs) as well as neutrophils are under circadian regulation, and the abundance of these critical immune regulators is affected by circadian disruption [61-63]. Thus, circadian rhythm disruption is a credible co-factor that can enhance alcohol-induced mucosal inflammation and carcinogenesis. Many individuals experience circadian disruption due to travel, irregular work schedules, or social jet lag (i.e.,

different sleep/wake cycles on the weekend vs. weekdays, present in at least 30% of western populations[64]). Delayed eating is an understudied life-style factor and yet the main energy contributor in industrialized countries is dinner, that is close to the rest time [24, 65]. Food timing is the main regulator of the circadian of the digestive tract [23, 66]. Our data from a mouse model of polyposis (Fig 1) indicate that wrong time (rest time) eating promotes alcohol-induced colon carcinogenesis. In this proposal, we will determine the effect of the combination (as well as each separately) of alcohol consumption and delayed eating on colonic mucosal inflammation, microbiota and markers of carcinogenesis in susceptible subjects for CRC. My Project will determine role of delayed eating in (**1**) exacerbation of alcohol-induced mucosal inflammation; **(2)** promotion of colon carcinogenesis after modest alcohol intake and elucidate the mechanism involved. My proposed studies will lead to identification of factors for risk stratification of at-risk individuals for CRC, and have implications to identify novel therapeutic targets.

Aim 1: To establish the "field" inflammatory profile associated with alcohol and wrong-time feeding-induced carcinogenesis

Rationale: We showed that circadian disruption in mice, using either genetic (*Clock*$^{\Delta 19}$ mutant) or environmental models (light:dark shift), promotes alcohol-induced intestinal inflammation [43]. Our data similar to prior studies [8, 9], show that wrong time eating causes intestinal circadian disruption in mice [Fig 2], and can augment alcohol's effect on colon carcinogenesis [Fig 1]. Here, we will do an in-depth analysis of mucosal and polyp immune profiles (using immuostaining and RNA-Seq), associated with enhanced carcinogenesis to alcohol and wrong time eating. The generated library will be used in Aim 3.

Scientific Impact: Identifying the mucosal immune profiles that mediate the enhanced carcinogenesis will be the basis of future mechanistic studies.

Educational Impact: PI learns immune interrogations by IF and RNA-seq, and analysis of generated data.

Experimental Impact: This aim will serve the human studies (Aim 3) using endoscopic samples, by providing an RNA-seq based signature in the field, predictive of colon carcinogenesis.

Methods: We will use already stored samples from alcohol fed and abnormal eating pattern controls (B6) and TS4CreAPC^{lox468} mice (both colon mucosal and polyp samples). The polyposis in TS4CreAPC^{lox468} mice occur mainly in colon, making it a superior model for CRC, compared to Apc$^{Min/+}$ with small intestinal polyposis. TS4CreAPC^{lox468} mice were fed standard chow *ad lib* during the dark (right-time) or during the light (wrong-time) on a standard 12h light:dark cycle for 3 months starting at 4 weeks of age. Alcohol (15%) and water were available during the feeding and non-feeding period, respectively. Mice were sacrificed at a single time (ZT0, the start of the light cycle) and intestinal tissue was collected for histology and RNA analysis. The feeding time (right vs. wrong time) did not affect overall food or water/alcohol intake, or weight of the mice. In **Aim 1a**, slides will be deparaffinized and subjected to immunostaining by immunofluorescent (IF), for CD4, FoxP3 and RORγt antibodies to determine the Treg (CD4+, FoxP3+, RORγt-), and Th17 (CD4+, FoxP3-, RORγt+) densities. We will use myeloperoxidase (MPO), as an acceptable marker for neutrophil infiltration[67], as we previously described[68, 69]. In **Aim 1b,** RNA-seq signatures for Treg/Th17, Neutrophils and their relative abundance will be inferred by deconvolution of gene expression profiles using CIBERSORT [70-73], and validated against the IF data to idenitfy signature in the mucosa field that is predictive of polpy profile [Fig 3]. Our data show that RNA-seq data is a reliable solution to distinguish immune cells, providing an accurate inflammatory signature, confirmed by gold standard (FlowCytometery) [74, 75](data not shown).

From the mouse RNAseq data we will use a linear model to generate continous gene expression signatures associated with alcohol, abnormal eating time, genotype, and pairwise interactions of alcohol, genotype and feeding time. These continous signatures will be submitted to gene set enrichment analysis (GSEA[76]) to be compared gene ontology categories and a range of experimentally-derived signatures (in MSigDB[77]), which will identify biological processes and other experimental perturbations, that will quantify the overall biological signal in the genes associated with each of the contrast in our model.

Anticipated results, potential pitfalls, alternative approaches and future directions: Our prelim data support our hypothesis that abnormal eating promotes alcohol-induced carcinogenesis. Our published data [42, 43, 78] support our expectation of seeing an enhanced mucosal inflammation due to alcohol and circadian dysrhythmia. In light of the key roles of Treg/Th17, and neutrophil signaling in CRC, we expect to see an imbalanced profile (e.g., a decreased Tregs, an increased Th17 response, increased neutrophils or an altered ratio) due to combined effects

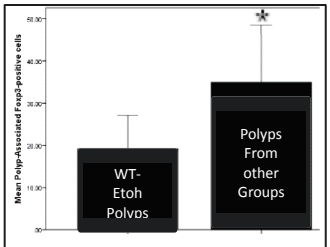

Fig 4. Polyp associated Tregs (FOXP3 positive cells) are significantly lower in the polyps of the "Wrong time + Etoh" group (left) vs. polyps from other animal groups (right). (*:p<0.05).

of ”wrong time” eating and alcohol. Our prelim data suggests Treg frequency as a credible immune candidate [Fig 4]. The data obtained in this Aim, can be the basis for further studies to dissect the underlying mechanism for the altered adaptive immune system induced by alcohol and how it is modified by abnormal eating patterns. It is possible that the altered IL17 response may be due to amplified responses from other IL17-expressing cells such as γδT cells as well as ILC3s, that we can analyze in the future. In the unexpected circumstance of not seeing any meaningful Treg/Th17/neutrophil signature associated with colon carcinogenesis, we will expand our analysis of the immune read outs to other components of innate immunity. For example, mast cells are important for colon tumorigenesis[79, 80]. Our group has reported that alcohol-fed mice develop expansion of mast cell and eosinophils in mucosa [36], a feature that is enhanced by circadian disruption. Thus, alternatively, we will study other innate immunity cells by analyzing mast cells, F480+ macrophages, Gr1 granulocytes, and NK1.1 cells. We will optimize our pro-tumorigenic profile by relating the innate inflammatory cell infiltration to the adaptive immune state and polyposis in our model. From the RNAseq and GSEA, we expect to define molecular systems associated with eating time and alcohol, and in conjunction with Aim 3 identify gene signatures most closely replicating alcohol or circadian interventions in humans. The gene signatures will also be useful in determing the validity of the animal model, by comparison to the differentially expressed genes identified in human RNAseq from Aim 3 and in confirming particular human oexpression modules are indeed asscoiated with altered circadian rythm or alcohol consumption.

Aim 2: To elucidate role of disrupted circadian homeostasis from delayed eating in promoting alcohol-induced carcinogenesis in susceptible individual.

Rationale: Circadian rhythms in intestine can be entrained by time of eating, independent of the central clock. Our preliminary data show that “wrong-time eating” does not affect central circadian rhythms as measured by activity of mice (data not shown), while disrupts colon circadian rhythms in mice (Fig 2). Thus, abnormal eating causes dyssynchrony of colonic circadian rhythm from the central clock. This should be exacerbated by alcohol intake because: 1) alcohol, even in small amounts, (0.5g/kg=2 glasses of wine/day for 7 days) can disrupt central circadian rhythms if combined with circadian disruption (induced by night shift work schedules) [Fig 5]; 2) moderate amounts of alcohol disrupt peripheral circadian rhythms (circadian gene expression in PBMCs) in both night- and day-shift workers [Fig 6]. These data show that moderate alcohol use can disrupt central and peripheral circadian rhythms, and that, central circadian disruption (due to night shift work) can promote alcohol’s effects; however, the effect of delayed food timing on these alcohol-induced effects is unknown. Here we will investigate the combined effect of alcohol and delayed food timing on central and digestive tract circadian rhythms in humans.

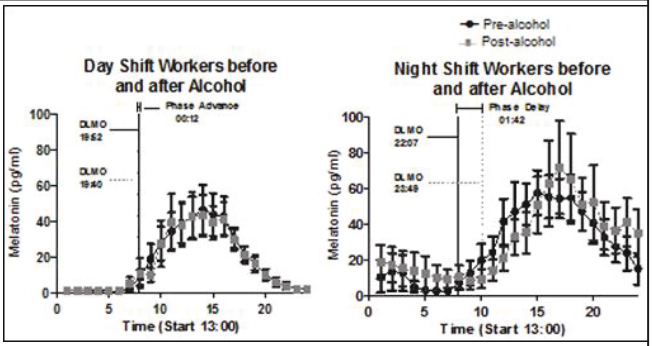

Fig 5. Shift of central circadian rhythm, measured by melatonin profile occurs in night shift workers, and not in day workers, after moderate alcohol.

Scientific Impact: Identifying the effect of delayed food timing, along with moderate alcohol intake on the human circadian system will have implications for preventive and therapeutic strategies for alcohol-induced CRC; our findings may also be relevant to other alcohol related pathologies, beyond cancer.

Educational Impact: The PI will learn interrogation of human circadian profile (to alcohol and food timing).

Experimental Impact: This Aim will serve to identify circadian disruption, as a mechanism explaining the link between delayed eating and alcohol-induced colon carcinogenesis.

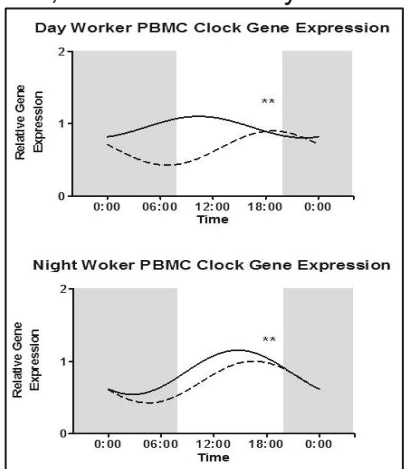

Fig 6. Peripheral rhythm aseesed by Clock gene expression in PBMCs before (solid line) and after (dotted line) alcohol, in day & night shifters.

Methods: Forty-four individuals (22 men and 22 women) with no history of alcohol use disorder [AUD] who have had advanced tubular adenoma in their sigmoid/descending colon within the last year will be recruited for a randomized cross-over experiment, testing alcohol use +/- two eating conditions on central and digestive tract circadian rhythms. To decrease diet variability of subjects, we will use a validated food frequency questionnaire (FFQ) to assess dietary habits over

the prior three months[81], to exclude subjects on special diets (e.g., vegetarian, gluten-free) and recruit individuals who meet criteria for a typical American diet consisting of daily fiber ≤ 16 grams and daily saturated fat ≥ 11 grams [82, 83].All subjects will be ≥ 21 years old [expected to be over 50 years old because of a history of polyps], and will be recruited after reviewing the endoscopy database of Rush University's Gastroenterology Division. Exclusion/inclusion criteria, definition of AUD and advanced adenoma are described in Human Subjects. Subjects are asked to avoid any alcoholic drinks for one week prior to each study. One week prior to the study, subjects complete the food/sleep questionnaire/(FSQ) (see Human Subjects) to record baseline food/sleep patterns. Sleep patterns will also be recorded by wrist actigraphy (Actiwach 2, Phillips Inc.) [84]. Each subject will undergo four conditions (each for one week in duration with a week wash-out in between [Fig 7]. The order of experiments will be randomized [concealed randomization]. A 24h food recall will be completed by participants using ASA24 at baseline and once during each intervention. This will be used to both determine that dietary intake is kept constant throughout the study, but also to verify time of eating on the FSQ in each of the treatment periods. "Right-time eating" is defined as breakfast (no later than 8am), lunch (no later than 1pm) and dinner (no later than 6pm), while "delayed eating" will be applied by delaying each meal by 3h. The choice of 3h is based on prior reports that alteration in the meal time for 3h can affect weight [12, 85]. Subjects will continue their regular energy and macronutrient content. All attempts will be made to keep sleep-wake periods constant between 11pm and 7am +/- 2 hours. The limit of 2 hours was selected because sleep/wake periods over 2 hours would lead to disrupted circadian rhythms [64]. Adherence to the protocol will be monitored via the FSQ. Subjects will enter the Biological Rhythms Research Lab after each week of the intervention period for 24h for assessments of central circadian rhythms (hourly salivary melatonin) and peripheral clock genes of the digestive tract from mucosal cells taken from buccal swaps once every 2h (12 time points). Extracted cells will be analyzed for expression levels of clock genes (*Per2, BMAL1, Clock, Cry1, Reverb-alpha, ROR-alpha*). While frequent endoscopic sampling of the intestine is not practical in humans, frequent buccal sampling is the most feasible method to assess the intestinal circadian rhythm [86]. Further, using buccal sampling is an acceptable method to assess the digestive tract rhythm, as the intestinal cells have common zeitgebers (SCN, food and GI hormones) [8, 87]. Nonetheless, we will also collect rectal swab every 12 hours (2 time points; first sample at the time of sigmoidoscopy) to validate that buccal sample represent disruption of digestive tract circadian rhythmicity by delayed eating. Our prelim data show feasibly of RNA analysis of clock genes from rectal swabs (Fig 8).The observed reciprocal RNA expression of the clock positive limb (*Bmal1* and *Clock*) vs. negative limb (*Per2* and *cry1*) members is suggestive of an expected anti-phasic pattern [88]. Rectal swab sampling more than twice is not practical. Although the rhythm of proximal part may phase-advance the distal part of the digestive tracts, rhythm of different segments of GI tracts are still in synchrony to maintain a healthy physiologic homeostasis[5]. Therefore buccal rhythm, though not a direct predictor of colonic rhythm, fits our purpose of assessing alteration of digestive tracts rhythm of subjects in response to our intervention (alcohol or food timing). For alcohol-related experiments, subjects will be given bottles of red wine and asked to drink wine with dinner (0.5 g/kg) for the week prior to the Lab visit. We have shown that this protocol causes disruption of circadian genes in PBMC [Fig 6] and promote systemic inflammation (unpublished data). To increase compliance, each subject is given enough bottles of wine to share with his/her partner at dinner.

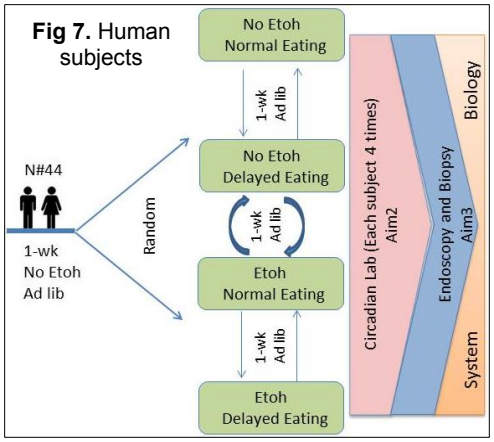

Fig 7. Human subjects

Anticipated results, potential pitfalls, alternative approaches, future directions: We hypothesize that delayed eating, while keeping calorie intake, food component, sleep-wake and activity levels stable, will promote alcohol-induced disruption of the peripheral circadian rhythm, and enhance central/peripheral circadian dyssynchrony. We expect delayed eating with alcohol to exhibit a phase shift in the expression of digestive tract clock genes (buccal mucosa) in relation to the central circadian rhythm assessed by the timing of melatonin rhythms relative to sleep (dim light melatonin onset, DLMO). Given our preliminary data, we expect to see alcohol effects on peripheral and central circadian rhythms. The expected enhancement of the deleterious effects of alcohol on circadian rhythms in humans by delayed/abnormal eating will have a significant impact beyond the cancer field, given the commonality of both habits, and the broad-spectrum of alcohol-associated diseases. It is possible that diet composition may affect circadian rhythms in the intestine.

Therefore we use FFQ at inclusion to minimize dietary variability among subjects. Prior reports indicate that individuals maintain a consistent dietary pattern over time and compensate for changes in energy intake over the short-term[89-91].Thus, it is expected that the subjects during our study period will maintain a consistent pattern of intake. In addition, the potential effect of the differences in energy and macronutrient intake at various time points throughout the day will be mitigated by the use of the cross over design. Further, we collect dietary composition data during the study period which will be used for post-hoc analysis of the potential modulating effect of type of diet on alcohol-induced effects. We selected a one-week period for each intervention as this time is sufficient to see changes in circadian rhythms in response to alcohol [Fig 6], and food [Fig 2] [8]. However we will re-evaluate this time selection following the first six subjects, and if, in the unlikely event we do not see any clear evidence of dyssynchrony, we will increase the intervention time to 10 days. One-week wash-out should also be sufficient to see the recovery from each intervention [8, 92-94]. Likewise, after the first six subjects, we will see if the wash-out period is adequate; if not, we will increase wash out times to 10 days. We will recruit 44 patients per our calculated sample size (see below). We plan to recruit 55 subjects, assuming 20% drop out (due to the length of the study). In our cross over study, every case acts as its own control, eliminating the need for large sample sizes that would have been required to account for inter-individual heterogeneity. Human circadian studies have a smaller sample sizes due to multiple time point data obtained for every subject, and the intensive nature of the protocol like ours with four of 24hr phase assessments in the Lab where subjects are kept awake. Indeed, a highly cited circadian study reported their findings with n of 5[95].

Aim 3: **To determine how disruption of circadian homeostasis by delayed eating promotes alcohol-induced colon carcinogenesis in susceptible subject.**

Rationale: Our group showed that shifts in the light:dark cycle exacerbate chemically induced colitis [42] and markedly increase intestinal permeability (particularly that induced by alcohol) [42]. Circadian disruption predisposes animals to multiple pathologies, likely through an immune-mediated mechanisms, and our animal data suggest an immune-mediated mechanism underlies alcohol-induced polyposis [36, 40]. Our data in humans indicate that circadian disruption (night shifts), increases alcohol-induced gut leakiness, a surrogate marker for overall gut inflammation, suggesting that circadian disruption could have synergistic/additive effect with alcohol on gut-inflammation [Fig 9]. Our preliminary data show that wrong-time eating in mice is associated with an enhanced alcohol-induced polyposis [Fig 1]. In Aim 3 we will determine the impact of delayed eating on alcohol-induced intestinal pro-tumorigenic inflammatory profiles (**Aim 3a**), associated with an enhanced carcinogenesis (**Aim 3b**) in at risk individuals for CRC (history of high risk adenoma). In **Aim 3c**,we will see if the inflammatory/proliferative effects of delayed eating patterns on epithelium, is mediated by dysbiosis of the intestinal microbiota [35] and is linked to circadian rhythm data in Aim 2 [96, 97]. The intestinal microbiota is increasingly recognized to be associated with susceptibility to CRC [98]. Dysbiosis is associated with a proinflammatory milieu, favoring development and progression of neoplastic lesions in the intestine. Recent lines of evidence suggest an effect of circadian rhythms on microbiota. Our group has shown that circadian disruption by the shift in light:dark cycle alters the microbiota, particularly when fed a high-fat, high-sugar diet (CRC promoting diet) [96]. Diurnal eating habits can affect microbiota, with wrong/rest time feeding inducing a phase shift of 12h in bacterial operational taxonomic units in mice, suggesting a direct effect of feeding time on microbiota [97]. Thus, we hypothesize that intestinal circadian dyssynchrony due to delayed eating alters the immune profile and facilitates colon carcinogenesis, at least partly, by altering colonic microbiota.

Scientific Impact: Identifying pathways responsive to delayed eating and alcohol will fill the gap in how disruption of circadian rhythms leads to a pro-inflammatory state and promotes alcohol associated pathologies.

Educational Impact: PI will learn human immune marker analysis by RNA-seq cibersorting, and a systems biology/network analysis approach for integrative data analysis.

Experimental Impact: Aim 3 will provide a basis for mechanistic studies to investigate causal links between alcohol, circadian system and CRC in future R01s by the PI.

Methods: Individuals recruited for Aim 2 will undergo unprepped sigmoidoscopy before admission to the Biological Rhythms Research Lab (4 times). Sigmoid mucosal biopsy specimens and stool are collected at baseline and at the end of each intervention week [Fig 7]. To eliminate circadian variation of mucosal markers, all biopsies will be collected at the same time of day (8-10am). To exclude the effect of bowel preparation on our measurements such as microbial composition (which is known to be

Fig 9. Increased intestinal permeability after 1 week of moderate alcohol use in Night workers was more than in Day workers.

affected by bowel-cleansing products) no bowel preparation will be used prior to sigmoidoscopy. All mucosal biopsies are collected from the sigmoid area (20cm from the anal verge) to minimize inherent regional differences among markers of interest. A total of 20 mucosal biopsies are obtained (n=8 RNA-seq, n=4 IHC staining, n=8 for future/alternative approach). We have taken 20 sigmoid biopsies / person in our prior studies in over 500 subjects with no complications. IHC will be done for Ki-67(mucosal proliferation as the early step in carcinogenesis), Caspase-6 (apoptosis) and gamma-histone-2AX (DNA damage). Several crypts per slide will be screened to calculate average densities of each marker to compare between pre and post-interventions in each subject. For IHC, sections of 50 microns will be cut and deparaffinized as we did previously [99-104]. RNA-seq samples taken from each patient will be interrogated in several ways. First, we will use CIBERSORT to infer the Th17/Treg/Neutrophils profiles in response to delayed eating, or alcohol, or both. We will use a gene coexpression approach applied to the RNAseq data to robustly define molecular systems whose activity is affected by alcohol and circadian interventions. Gene coexpression is based on synchronized gene expression across multiple samples; it represents the activity of many regulatory mechanisms, shows tissue-specific results and has the robustness of pathway-level results[105]. It is particularly appropriate for the emphasis on this project on synchronization, because gene coexpression is based on synchronized expression and represents biological processes across time and tissue location in an organism[106]. This approach allows us to focus on robust biological processes active in the tissue, through "coexpression modules" which are groups of transcripts[107] to see how these are related to alcohol and circadian rhythm.

Coexpressed gene modules will be identified for RNAseq data acquired at the beginning and end of each trial condition. The predominant function of each module will be assessed through enrichment of GO categories. We will prioritize the coexpression modules that are enriched in differentially expressed genes (between control and alcohol/circadian-perturbed states) or correlated with any traits of interest (e.g, dysbiosis). We will use permutation tests to identify modules that experience a significant gain or loss of correlation by the end of a trial period, or between trial periods. In addition to these module tests, we will also use tradition differential expression and linear models, to identify differentially expressed gene signatures between all conditions and the GO functions enriched in those genes. (Fig. 10)[108]. The gene members of these modules and their biological function will be contrasted with the gene sets from mouse models through module overlap statistics[109] and by rank tests of differential expression to determine the extent to which the mouse model captures the overall extent of the genes affected in humans by alcohol and circadian dyssynchrony. Thus, as we have demonstrated in the context of other diseases[110, 111], our coexpression methodology will comprehensively extract gene sets related to diverse aspects of circadian disruption or alcohol.

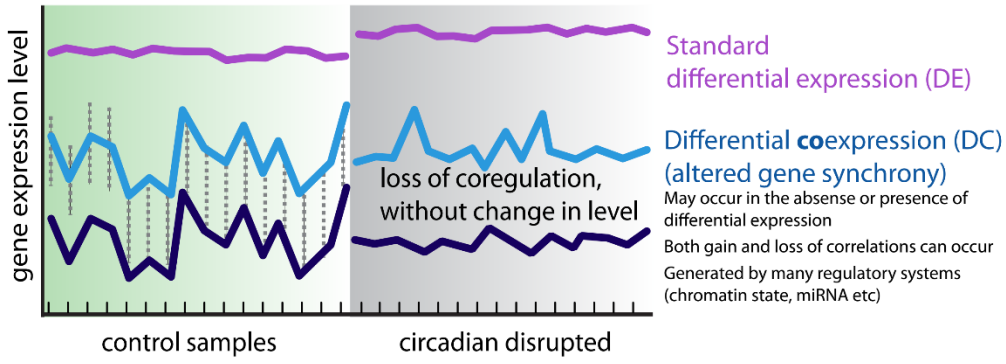

Figure 10. Altered gene synchronization provides additional information about biological regulation beyond traditional differential expression. Gene-gene correlations that are condition-specific (i.e. only found in circadian disrupted samples) provide clues to the biological effects of altering circadian rhythm. Specifically, coexpression modules with many such state-specific correlations may be important for transducing circadian and alcohol effects.

For microbiota analysis, we examine stool microbiota collected at the time of sigmoidoscopy using 16S rRNA generated gene amplicons. We will identify species whose abundance correlates with average expression of coexpression modules, which are potential drivers of that gene set.

Anticipated results, potential pitfalls, alternative approaches, future directions: We expect that delayed eating + alcohol consumption will be associated with imbalance in Th17/Treg/Neutrophil profiles, mucosal hyperproliferation, increased proliferation rate, and reduced apoptosis. Such observations would support our overall hypothesis that delayed eating in subsets of alcohol users, worsens circadian disruption associated with a pro-carcinogenic inflammatory milieu, which if not addressed for months or years will promote tumorigenesis. It is possible that circadian disruption, in addition to alterations in selected immune markers, or independent from them, enhances alcohol-induced carcinogenesis via other alcohol-related mechanisms, such as oxidative stress. Therefore if the immune pathways are not involved in mediating circadian effects, markers of

additional/alternative pathways (e.g., iNOS and NF-kB for oxidative stress) could be examined in the future. Besides Treg/Th17 and neutrophils, global RNA gene expression can be regressed against outcomes in this study, further refining pathways that are associated with eating pattern, alcohol, or their combination. From the RNAseq coexpression analysis we expect to define major biological processes which are the substrates and downstream correlates of altered circadian rhythms. We expect the altered immune profile to correlate with mucosal proliferative profiles and dysbiosis. We expect these changes to correlate with circadian dysrhythmia, measured in Aim 2. But in the event there is no relation between circadian disruption and mucosal markers, other mechanisms may mediate the effect of delayed eating on alcohol-induced tissue injury. For example, metabolic pathways (e.g., mTOR) are linked to CRC and are entrained by food [112-114], and can be investigated by gene expression profiling of the biopsies obtained in this Aim. This Aim will let us generate an integrated map of the immune response to delayed eating, alcohol and circadian dyssynchrony. Results from the microbiome analysis will identify the upstream signals that can signal the upregulation or downregulation of genes and pathways that modulate the inflammatory response. Although it would be ideal to study pre-cancerous lesions/polyp, we cannot subject polyps to RNA analysis as the histology of the entire polyp must be reviewed by a clinical pathologist to exclude any possible cancerous changes. On the other hand, cancer is the end product of accumulating events and will not be appropriate for our purpose of identifying early signatures for alcohol induced carcinogenesis. We will therefore profile biopsies from "normal" appearing mucosa", but from the tissue of a subject that recently harbored an advanced adenoma and thus their sigmoid mucosa is expected to have a "field defects" . Prior studies have shown that abnormal mucosal signatures in patients with CRC are field defects and are present in the normal appearing colonic mucosa [115-117]. To maximize the field effect in our subjects with advanced adenoma, we will recruit those with sigmoid lesions in whom the abnormalities are expected to occur in our biopsy field. Biopsy materials contain a small number of immune cells. Thus, we will use Cibersorting of RNA-seq data to identify immune profiles associated with delayed food timing and alcohol. Our data show that RNA-seq data from Cibersorting analysis is feasible to identify impact of circadian disruption on immune cells [Data not shown]. Alternatively, we will perform immunostaining of sigmoid biopsies for our immune candidates which can also be used for validation of the Cibersorting. As in Aim 2, we will do an interim analysis after the first 6 subjects and prolong the intervention duration if 1 week is insufficient to see changes in the mucosal readouts. Prior studies showed that type and time of diet rapidly (<1 week) alter the gut microbiome[97, 118]. Coexpression analysis will identify coexpressed gene sets which gain or lose correlation under alcohol and/or circadian perturbations in humans associated with other traits measured in each subject such as dysbiosis and mucosal proliferation. Subset of these coexpressed gene sets may be enriched in differentially expressed genes from mouse disease models in Aim 1, which will establish key components of those models and identify what aspects of human effects they best represent. Collectively these systems-level results will establish the major biological processes associated with specific components of alcohol and circadian disruption.

Statistics and Overview of data analysis. A sample size of 44 achieves 81% power to detect 0.1 mean changes in circadian rhythm profiles measured by melatonin and circadian gene expression and changes in immune gene expression and carcinogenesis markers (proliferation, apoptosis and DNA markers) within a subject with an estimated standard deviation of differences of 0.3 under a significance level (alpha) of 0.05 using a two-sided paired t-test. Power will be increased by using multivariable regression including other covariates that may affect the outcomes including demographics such as gender, BMI, age, race and chronotype. Repeated Measure ANOVA with two factors (alcohol, eating pattern) and their interaction (alcohol*eating) will be used. To control various types of additional predictors other than alcohol and eating conditions, a Generalized Estimating Equation (GEE) model approach will be used. **I. Generation of immune profiles in response to alcohol and abnormal food timing:** In Aim 1**,** RNA-seq, will be done to infer Treg/Th17/neutrophil profiles, associated with worsening polyposis in response to alcohol and abnormal eating patterns (or each alone). The immune signature will be used for profiling the gene expression data from mucosal biopsy tissues obtained in Aim 3a. **II. Immune profiling from sigmoid tissue**: In Aim 3a, RNA-seq will be done on biopsies followed by immune profiling using CIBERSORT [72, 73]. Immune signatures determined in Aim1a will be used as a guide to interrogate the profiles obtained from endoscopic sampling in response to alcohol or delayed food timing, or both. **III. Microbial composition and structure**: 16S rRNA amplicons will be sequenced using an Illumina MiSeq instrument, with an anticipated output of 50,000 sequences per sample. The surveys of microbial genes generated by deep-sequencing will be used for microbial composition profiling using the Bioconductor R package metagenomicSeq and diversity analysis with the Shannon's index using the

R package Vegan [119, 120]. **IV. Correlation with Study outcomes**: Differential RNA-seq gene and pathway signatures will be regressed against outcomes in this study separately: the mucosal markers (Aim3b), dysbiosis (Aim3c) as well as circadian rhythm profile (Aim2). This will refine the pathways that are associated with each eating pattern, alcohol, or their combination. The microbiota data will be correlated with the RNA-seq data in Aim3a to determine gene correlates of alterations in immune (Th17/Treg/Neutrophils) profiles in response to study variables (alcohol and food timing) with microbiota population complexity and abundance. **IV. Integrated analysis:** Using an integrated systems biology/network analysis approach[121-123], we will look for interactions among immune profiles generated by gene expression data (cibersorting) and microbiome signatures against epithelial proliferations markers as well as circadian measurements (Aim2). Bioinformatics workflow uses the R [www.r-project.org] and Bioconductor[124] platform, and the public databases MsigDB[76] InnageDB[125]. This integrative approach will enable us to identify the effect of eating patterns, in individuals who drink alcohol, on CRC-relevant inflammatory profile and CRC markers, and can interrogate the role of microbiota as a possible mediator. Incorporating data from Aim 2 into our model, we study the role of central-intestinal circadian dyssynchrony induced by alcohol and delayed eating as the driver of the anticipated outcomes in Aim 3.

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4. PROTECTION OF HUMAN SUBJECTS.

4.1.1 Risks to Human Subjects

a. *Human Subjects Involvement, Characteristics, and Design*

Forty-four subjects will be recruited into the study. These subjects will undergo a full history and physical examination by the PI, a licensed physician, to determine their eligibility and co-morbidities. Subjects will then sign an informed consent. In addition, subjects then fill out a questionnaire regarding their demographic data, health status, symptoms, alcohol use, dietary habits, and sleep schedule.

At baseline and prior to each study visit, subjects will complete a sleep and food diary by completing the FSQ (food timing/sleep questionnaire-attached), food frequency questionnaire (FFQ) and a standardized 24h food recall. The latter will be used to determine whether dietary intake is changed throughout the study, and to verify time of eating on the FSQ in each of the treatment periods. Subjects would also be interviewed by the PI and a clinical coordinator using the LDH assessment tool to carefully evaluate their alcohol drinking history in order to exclude subjects with alcohol use disorder (AUD). AUD will be determined according to NIAAA criteria for alcohol abuse or dependence & DSM-IV criteria for alcoholism. We will not include AUD subjects because: 1) the mucosal markers of heavy drinking could be irreversible and not comparable among different conditions of food timing and alcohol use; 2) heavy drinking is associated with multiple organ dysfunction that could independently affect CRC risk, thus exerting cofounding effects; 3) heavy drinking has profound effects on sleep and circadian patterns that may not be reversible by a sobriety period of only one week. We also will exclude patients with active cancer, as cancer itself can affect the behaviors and circadian rhythm [1-2].

We will include both men and women, since we predict a possible difference between female and male subjects in response to eating pattern, and alcohol, due to known effects of gender on alcohol metabolism as well as on mucosal immunity [3-4]. We will recruit patients with a history of high risk adenoma for several reasons: 1) Our preliminary data supports the synergistic effect of abnormal eating time and alcohol in a susceptible host (APC-mutant mice) and not in Black 6 mice. 2) Germline *APC* mutation occurs in patients harboring the trait for familial polyposis syndrome (FAP). However, this group comprise a very small portion (≤1%) of CRCs in general, and usually undergo prophylactic colectomy at an early age to prevent cancer formation. On the other hand, *APC* is the most frequently mutated gene in sporadic pre-cancerous colon lesions (>60% in tubular and tubulovillous adenomas) and in cancerous lesions (>90%) of the colon. 3) If my study fails to show any effect of eating pattern and/food timing on alcohol's effect on colon carcinogenesis in susceptible hosts, I won't need to test this hypothesis in healthy subjects. We will include patients with a history of a conventional type of adenoma (tubular, tubulovillous and villous), which are the most common colonic precancerous lesions [with frequent *APC* mutations], as immediate follow up to my animal studies in *APC*-mutant mice. In the future, I will extend my studies to individuals with serrated adenomas, in which *BRAF* or *KRAS* mutations more commonly lead to colorectal cancer [5]. The serrated polyps account for a minority of colorectal polyps found during endoscopy (1/5 to 1/10 of tubular adenoma rates). The distinct molecular subtypes of polyps may represent exposure to different environmental risk factors. For example, alcohol in a few but not in all studies is suggested to be associated with serrated polyps. Therefore, as an alternative strategy, and/or in my future studies, I will include patients with a history of serrated polyps.

Each subject will experience four conditions (each for one week in duration with a week +/- 2 days wash-out in between): (1) "right-time eating" / no alcohol, (2) "right-time eating" / with alcohol, (3) "delayed-eating" / no alcohol, (4) "delayed-eating" / with alcohol. The order of experiments will be randomized [concealed randomization]. All subjects will undergo unprepped sigmoidoscopy after each week of intervention. In Aim 2, all subjects will have an option to undergo a 24h circadian assessment in the Biological Rhythms Research Lab after each week of intervention. We will assess (i) central circadian rhythms by collecting hourly salivary samples for melatonin assays and (ii) peripheral rhythm in the intestinal tract by buccal swabs once every 2h (12 time points) as well as by rectal sampling twice (every 12 hr). For Aim 3, sigmoidoscopy without sedation will be used to obtain colonic samples as the safe method compared to colonoscopy, which has some small but finite risks associated with the procedure (e.g, bleeding or perforation) as well as sedation.

Subject characteristics and inclusion and exclusion criteria:

Method: We will recruit 44 individuals (men and women) with no history of AUD (AUD = anyone meeting 2 out of 11 criteria according to DSM–5) but who have had advanced tubular adenoma in their sigmoid/descending colon within the last year into a randomized cross-over experiment. All subjects will be adults (greater than – 21 years old [expected to be over 50 years old because of history of polyps]), and will be recruited after reviewing the endoscopy database of the Rush University Gastroenterology Department.
The PI will exclude individuals with: **1)** a known genetic predisposition to CRC (e.g., FAP, Lynch syndrome); **2)** a history of CRC or inflammatory bowel diseases; **3)** presence of comorbidities that might effect the circadian system such as chronic renal failure (creatinine>1.2 mg/dL), cirrhosis, advanced neurological conditions [e.g. Parkinson's disease, MS, epilepsy], psychological disorders including PTSD and major depression (score ≥ 15 on the Beck Depression Inventory), sleep apnea (score of being at high risk in ≥ 2 or more categories on the Berlin Questionnaire) [6], restless leg syndrome (score ≥ 15 on the IRLS Study Group Rating Scale [7]), inpatient status, advanced cardiac failure (NY classification stage III/IV), night shift workers with active shift work in the past month, or planned shift work that will occur during the study, and people who crossed more than two time zones in the previous week; **4)** conditions that alter or necessitate a particular eating pattern such as uncontrolled diabetes (Hgb-A1c>8%), or eating disorders (such as nocturnal eating syndrome); **5)** Conditions that alter the microbiota [infection or recent history of antibiotic use within three months, or use of pro- or pre-biotics within one month prior to recruitment; **6)** regular use of medications that can potentially affect melatonin profiles such as melatonin, metoclopramide, psychotropic medication, and hypnotics during the four weeks prior to the study; **7)** Large inherent differences in alcohol metabolism [therefore, in this proposal we will not include Asians due to common polymorphisms of enzymes involved in alcohol metabolism]; **8)** Any active cancer; **9)** inability to sign an informed consent; and **10)** Subjects who do not drink alcohol. Regular use of any medications that alter mucosal inflammatory profiles such as NSAIDs or COX inhibitors will be stopped for one month prior to the study initiation. Those in whom stopping NSAID is not clinically safe will be excluded. Low dose aspirin [80 mg] is allowed.

Human safety issue:

Alcohol is commonly consumed in society. Delayed food timing, defined as large dinners close to the bedtime, is another very frequent occurrence in today's 24 hour society. We are testing interactions between two factors that are already occurring commonly in our society today. Therefore we are not imposing any new risks to the participants due to the short duration of the exposure (one week) to moderate alcohol, and/or a 3 hour delay in food timing.

b. Sources of materials.

Subjects (n= 44) will be primarily recruited from the review of our endoscopic database including all patients that underwent colonoscopy within the past year and were found to have advanced adenomas according to the American Association of Gastroenterology guidelines in their /descending colon area. The search for candidates will continue during the award period by reviewing the colonoscopy results of the patient who undergo colonoscopy at Rush Gastroenterology Department. Advanced adenoma is defined as adenomas 1 cm or greater, or with advanced neoplastic features per pathology review including presence of villous components (tubulovillous or villous), or high-grade or severe dysplasia. Patients will be contacted by a clinical coordinator and will be invited to participate in the study.

Subject data.

All subjects will be interviewed by the PI and a clinical coordinator. Those who fulfilled the screening criteria will be invited, interviewed & examined by the PI to establish compliance with inclusion/exclusion criteria. This will include a detailed history of past & current complaints & a complete physical exam. History of alcohol consumption will be assessed by a validated, NIAAA-endorsed assessment instrument-The Lifetime Drinking History (LDH). All subjects: **1)** will fill out detailed questionnaires so that we can collect demographic data, data on their alcohol consumption, medical histories, and information on diet; **2)** will be interviewed (using LDH instrument) by the PI and a clinical coordinator; **3)** will fill out the FFQ[8] and FSQ questionnaire as well as the 24 ASA at baseline (and after each intervention) as described below; **4)** will undergo an exam by the PI and will be randomized (concealed randomization) to experimental groups; **5)** will undergo an unprepped unsedated flexible sigmoidoscopy and optional 24 hour circadian analysis that includes mouth swabs every 2

hours (for buccal RNA extraction) and salivary melatonin done every 60 minutes. We will also collect rectal swab every 12 hours (2 time points); first sample to be taken at the time of sigmoidoscopy.

Diet and sleep assessments:

Although the primary aim of this study is not to see the effect of type of diet on our outcomes, it is possible that the type of food affects the circadian rhythm, immune response and microbiota and eventually the measured carcinogenesis markers. Therefore, to decrease diet variability of subjects, we will use a validated food frequency questionnaire (FFQ) to assess dietary habits over the prior three months, to exclude subjects on special diets (e.g., vegetarian, gluten-free) and recruit individuals who meet criteria for a typical American diet consisting of daily fiber $\leq$ 16 grams and daily saturated fat $\geq$ 11 grams. We will use the ASA24, a standardized 24h food recall questionnaire, which will be completed by participants at baseline and during each intervention, prior to each study visit. This will be used to determine whether dietary intake is kept constant throughout the study.

In addition we will use a structured, IRB-approved questionnaire (FSQ) that was developed by our group to assess the timing of food intake as well as alcohol consumption. The time of eating obtained from the FSQ will be verified by the ASA24, a standardized dietary assessment tool. The FSQ also assesses sleep patterns. The sleep pattern will be further verified by Sleep patterns recorded by wrist actigraphy (Actiwach 2, Phillips Inc.). FSQ, ASA24 and wrist actigraphy will monitor adherence to the protocol.

Dim Light Melatonin Onset and clock gene expression.

A phase assessment in the laboratory will be conducted to measure each patient's endogenous melatonin profile. Patients will be seated and kept awake in recliners in dim light (4100 K, <5 lux, at the level of the patients' eyes, in the direction of gaze, Minolta TL-1 light meter, Ramsey, NJ). Starting at 14:00, patients will give a saliva sample every 60 minutes for 23 hours using Salivettes (Sarstedt, Newton, NC). Laboratory staff will continuously monitor all patients throughout the phase assessment session. Toothpaste and mouthwash are not allowed during the phase assessment. Fluids will be permitted, except in the 10 minutes before each sample, and patients are required to rinse and brush their teeth with water while remaining seated 10 minutes before each sample if they consume a drink. The saliva samples will be obtained every hour and centrifuged immediately after collection and frozen. The samples will later be shipped in dry ice to Pharmasan Laboratories (Osceola, WI) and will be radioimmunoassayed for endogenous melatonin levels. All samples from an individual patient will be assayed in the same batch. Mouth and rectal swabs will be obtained every 2 and 12 hours respectively and immediately put in freezers. RNA from the tissues will be extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. To further ensure integrity of RNA samples, we will incorporate an extrapurification method, using the Qiagen RNeasy Clean-up kit (QIAGEN, Valencia, California). Total mRNA quantities will be quantitated and amplified cDNA will be generated through reverse transcription, using using SuperScriptTM II Reverse Transcriptase (Invitrogen) and poly(dT)15 primers (Roche). The resulting individual cDNA levels for all clock genes will be quantified on the ABI 7900 Real Time PCR machine, using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California). All the copy numbers will be normalized using the expression level of a housekeeping gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Urinary Melatonin

To assess for central circadian rhythm, primary urinary metabolite, Overnight 6-sulphatoxymelatonin (aMT6s) excretion levels from the first morning void will be collected in one bin at home, after each week of treatment condition. The morning void as well as any possible additional nighttime voids will be collected in one jug . Samples will be brought the same morning to our facility when pt comes for sigmoidoscopy. Urinary 6-sulfatoxymelatonin will be assessed through a competitive enzyme-linked immunosorbent immunoassay and adjusted for urinary creatinine levels to control for urine volume.

4.1.2 Adequacy of Protection Against Risks

a. Plan of recruitment and consent.

The initial step in recruitment of subjects will be by reviewing our endoscopy database for the past year, and during the award period to identify potential candidates with a history of advanced adenoma. More than

11,000 colonoscopies are done annually at Rush. It is expected that at least 5% of the individuals would have advanced adenoma in general, and at least 50% of those are located in the sigmoid/descending colon. Therefore we estimate to have 275 potential candidates with advanced adenoma in the past year and then each year of the study period for the total of 1375 (considering the past year and first 4 years of the award period) that we can screen, contact and invite for the study. After initial screening per chart review, patients will be contacted by the PI and a clinical coordinator and will be invited to participate in the study. Written informed consent will be obtained by the PI after the study is clearly explained to the subject. The recruitment plan, study plan and consent form will be approved by the Institutional Review Board at Rush.

b. Protection against potential risk(s).

My primary mentor has been doing clinical research using alcoholic subjects for over two decades. The only potential risk for most subjects is minimal discomfort from fatigue during the DLMO assessment. As stated in the "human safety issue" we are not imposing any new risks to the participants due to the short duration of the exposure (one week) to moderate alcohol, and/or a 3 hour delay in food timing, both common lifestyle habits. Unsedated sigmoidoscopy to obtain colonic mucosal samples may be associated with minimal discomfort of the subject, but would not carry procedure-related risks. The alternative to the study is not to participate. It will be made clear to all potential subjects that choosing not to participate in the study will not affect their health care.

Procedures to minimize risks.

An experienced clinical coordinator who has extensive experience in dealing with research subjects and in obtaining information through questionnaires will help the PI eliminate any discomfort that might be perceived by the participants. The PI is a board-certified gastroenterologist with experience in performing endoscopic procedures including sigmoidoscopies and will perform all the procedures. The biopsies will be taken by cold forceps that may cause minimal mucosal bleeding (~1 cc), but per se are not associated with any pain/discomfort. All attempts will be made to prevent disclosure of subjects' demographic data by using research codes for subject identification. Subjects' information will be coded and kept in a secured database.

4.1.3 Potential Benefits of the Proposed Research to Human Subjects and Others

Justification of the risks.

There is minimal risk involved in participation in the study and these risks are related to discomfort with circadian assessment and sigmoidoscopy.

Importantly, the knowledge gained from the proposed study could have huge implications for CRC prevention in alcohol consumers. Most immediately, individuals in our study who are found to have the most dramatic responses to alcohol and delayed food timing could be advised to follow a circadian-friendly based food timing or chronotheraputic regimen.

4.1.4 Importance of Knowledge to be gained

Defining patients at risk for alcohol-induced CRC and intestinal damage.

4.1.5 Data and Safety Monitoring Plan

Adverse event monitoring and reporting: Patients will be asked to record each adverse event and report each adverse event at study visits. The PI will monitor adverse events at the time of clinical visits by pointed questions.

Definition and recording of adverse events: An adverse event is any untoward medical occurrence experienced by a patient. An event may consist of the development of a disease, a set of related symptoms or signs, or a single symptom or sign.

Data Safety Monitoring Board (DSMB): Ongoing safety monitoring for the study will be carried out by a DSMB that will be established specifically for this study. This board will consist of 3 independent safety and data consultants who are physicians and scientists at Rush University Medical Center. DSMB consultants will meet every six months and receive up to date reports from the clinical coordinator.

Notification of Serious Adverse Events: The date, time, severity, duration, and description of adverse events will be recorded by the PI on an adverse event reporting sheet and reported to the DSMB and the IRB immediately. Subjects with clinically significant symptoms or physical findings will be followed until resolution. Any significant laboratory test result will be followed until it has returned to baseline. Any adverse event of a nature serious enough to warrant withdrawal from the study will also be reported to the DSMB immediately.

4.2 Inclusion of Women and Minorities

This study will recruit subjects of both genders. We anticipate that we will use samples from an equal number of female and male subjects in this study.

All ethnic and racial groups will be recruited except for Asians. We plan to exclude Asians from the study because of the possible confounding effect of a different polymorphism of enzymes involved in alcohol metabolism in this racial group. We anticipate that racial distribution of our study group samples will be similar to our local population with 30% African American, and 70% Caucasian and the ethnic distribution of Caucasians will be 10% Hispanics.

4.4 Inclusion of Children

We include no children (<18yo). Anyone younger than 21 will be excluded as they cannot legally consume alcohol. A Majority of subjects are expected to be over 50 years old because of the study's requirement of a history of polyps. We will exclude subjects older than 70 years due to increased frequency of other medical problems / complications that may affect intestinal function.

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