Disrupted Diurnal Oscillation of Gut-Derived Short Chain Fatty Acids in Shift Workers Drinking Alcohol: Possible Mechanism for Loss of Resiliency of Intestinal Barrier in Disrupted Circadian Host

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Abstract

Microbiota derived short chain fatty acids (SCFAs) are produced by fermentation of non-digestible fiber, and are a key component in intestinal barrier homeostasis. Since the microbiome has diurnal fluctuations, we hypothesized that SCFAs in humans have a diurnal rhythm and their rhythmicity would be impacted by the host central circadian misalignment (night shift work) which would make intestinal barrier more susceptible to disruption by alcohol. To test this hypothesis, we studied three groups of subjects: patients with alcohol use disorder, but no liver disease (AD), healthy day workers (DW), and night workers (NW). All subjects were studied at baseline and then in DW and NW subjects after moderate daily alcohol (0.5 g/kg) for seven days. Gut derived plasma SCFAs showed a significant circadian oscillation by cosinor analysis in DW; however, SCFA in the AD and NW subjects lost 24 hour rhythmicity. Decrease in SCFA correlated with increased colonic permeability. Both chronic and moderate alcohol consumption for one week caused circadian disruption based on wrist actigraphy and urinary melatonin. Our study shows that: (1) gut derived plasma SCFAs have a diurnal rhythm in humans that is impacted by the central clock of the host; (2) moderate alcohol suppresses SCFAs which was associated with increased colonic permeability; and (3) less invasive urinary 6-SM correlated and rest-activity actigraphy correlated with plasma melatonin. Future studies are needed to examine the role circadian misalignment on gut derived SCFAs as possible mechanism for loss of intestinal barrier resiliency to injurious agents like alcohol.

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Keywords

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Introduction

The human circadian clock is controlled by the central or master clock, located in the suprachiasmatic nucleus (SCN) which coordinates the timing and synchronization of peripheral clocks that are found in every organ in the body. As organisms evolved based on the 24-hour periodicity of the planet"s natural light-dark cycle, pacemakers like the SCN have been tightly regulated across species and are essential for health.(1) The central clock controls sleep-wake cycle and the transcription of ~ 80% of protein coding genes in the primate brain and peripheral tissues.(2) Many of the genes that are under circadian control are involved in metabolism, inflammation, and oxidative stress; and therefore not surprisingly misalignment between central clock and environment has been associated with obesity,(3) metabolic syndrome,(4) chronic inflammatory conditions,(5–7) and cancer.(8–10)

The best-studied model of chronic circadian disruption is in shift work. Data from 2010 shows 29% of US workers worked an alternate or non-day shift,(11) and 15% of workers, approximately 23 million people, reported regularly working the night shift. In shift workers, exposure to artificial light at night causes misalignment of the circadian timing or the term „circadian disruption."(12) In addition to shift work, other life style habits, light at night through TV and electronic devices (smart phones/PC/Laptop), and irregular eating and sleep/wake habits promote circadian misalignment. Thus, detailed mechanism of how circadian misalignment affect normal biology/physiology of the host is a critical unmet need because of its prevalence of circadian misalignment. In addition, shift work has been linked to breast cancer,(8) cardiovascular disease,(13) metabolic syndrome,(14) and irritable bowel syndrome(15). Combination of lifestyle factors may be important, as the Nurse"s Health Study recently found that night shift work was associated with an increased risk of diabetes in those individuals with unhealthy lifestyle factors such as alcohol consumption.(16) The most reliable measure of central circadian timing in humans is the onset of melatonin secretion, when measured in dim light conditions (dim light melatonin onset, DLMO).(17) Melatonin typically begins to rise 2–3 h before the onset of sleep, and allows for frequent and precise sampling in saliva or plasma. Whereas the main urinary metabolite 6-sulphatoxymelatonin (6-SM) correlates well with salivary or plasma melatonin and can be measured at home but lack the precision of an in lab DLMO. Several studies have shown no or minimal impact on DLMO with alcohol.(18,19); however, in our previous work, we showed that disruption of circadian rhythms increased alcohol-induced colonic permeability and endotoxemia, (20) suppressed plasma melatonin,(21) and that night workers (NW) have phase delay compared to day workers (DW) after moderate alcohol consumption.(20) However, the mechanism of increased susceptibility to alcohol injurious effects on intestine in circadian disrupted host is not known, We and others have shown that alcohol consumption disrupts normal microbiota composition and function(22–24) and this alcohol-induced dysbiosis is characterized by increase abundance of putative pro-inflammatory

bacteria and decreased abundance of putative anti-inflammatory bacteria like SCFA.(25) Several studies have shown that SCFAs, especially butyrate, is required for maintaining healthy intestinal barrier and low butyrate has been associated with disrupted intestinal barrier integrity.(26,27) It is now also well established that the microbiota exhibit a diurnal rhythm,(28) and the circadian rhythmicity of the host can influence the intestinal microbiota structure and function.(28–31) It is intriguing that dysbiosis in disrupted circadian rodents and human is also characterized by decreased abundance of SCFA producers like *Lachnospiraceae* (phylum *Firmicutes*, class *Clostridia*).(32) These data suggest that the increased susceptibility of the intestine of the disrupted circadian host to alcohol may be through dysbiotic intestinal microbiota.

Therefore, there is a clear rationale to assess plasma SCFAs, which correlate with stool SCFA,(33–35) over 24 hours in individuals with and without circadian misalignment and to determine the impact of alcohol consumption. We hypothesized that plasma SCFAs would have diurnal rhythmicity and would be impacted by central circadian disruption (night shift work) and disruption of SCFA diurnal oscillation might be a mechanism for increased susceptibility of shift workers to alcohol-induced hyperpermeability that we have previously reported.(20) Accordingly, we measured plasma SCFAs, wrist actigraphy, and urinary 6-SM in three groups of subjects: patients with alcohol use disorder (AD) who had no clinically significant liver disease, healthy, day (DW), and healthy night shift (NW) workers with the DW and NW subjects were studied before and after moderate alcohol (0.5 g/kg, red wine) for one week.

Methods

Participants

Participants were recruited through advertisements, posted flyers, or self-referral. All subjects gave written informed consent, and all procedures were approved by the Rush University IRB – #10012604, The study was designed to include three groups: AD, DW, and NW. The AD group was established via the Lifetime Drinking History (LDH) questionnaire and both the DW and NW groups did not fit criteria for AD by LDH. The DW cohort was comprised of healthy subjects on a stable work schedule of 7am-7pm for at least 3 shifts a week for a period longer than 3 months. The NW group consisted of healthy subjects on a stable work schedule of 7pm-7am for at least 3 shifts a week for a period longer than 3 months. All subjects completed blood tests and questionnaires at their initial visit and were excluded if they met any of the following criteria: **1)** Any history of or blood tests consistent with liver disease (LFTs > 1.5 normal), renal impairment (Creatinine > 1.5), cardiovascular disease, diabetes (Hgb-A1 >8%), or thrombocytopenia (<80k); **2)** Major depression (score 15 or any endorsement of suicidal intent on the Beck Depression Inventory);(36) **3)** Sleep apnea (score high risk ≥ 2 or more categories on the Berlin Questionnaire);(37) **4)** Restless leg syndrome IRLSSG consensus criteria for Restless Leg Syndrome;(38) **5)** Regular use of medications that affect melatonin – including beta blockers, psychotropic medication (ie SSRI), and exogenous melatonin products.

Baseline Measures

After a baseline questionnaire assessment and blood tests, all subjects were asked to keep their usual sleep schedule during the week prior to the 24-hour circadian phase assessment in the Clinical Chronobiology Center (**CCC**). After the first phase assessment, DW or NW subjects were given red wine, a 2013 Bota Box Malbec, alcohol 13.5%, origin Mendoza, Argentina, and instructed to drink 0.5 gram of Etoh/kg of body weight each day for the 7 days prior to the second 24-hour phase assessment – for the majority of subjects, this meant 2–3 glasses of wine a day. Subjects were instructed to drink the wine after finishing work and prior to bedtime. Subjects in the NW and DW cohort were asked to keep a diary the week of alcohol consumption so as to record the time they drank the alcohol. The AD subject cohort was not given alcohol and only underwent the initial circadian phase assessment. During each phase assessment, subjects were kept in dim light (<5 lux, measured every 2 hours with a Minolta TIL-1 light meter), and seated in a reclining chair at a constant posture. Subjects were given small, nutritionally balanced snacks instead of meals and not allowed to exercise during the phase assessments to limit the impact of these factors on circadian timing (constant routine).

Plasma Melatonin and Intestinal Permeability

Plasma melatonin was collected hourly for 24 h between 1300 and 1200, for a total of 24 samples. Patients were kept in dim light (<5 lux, measured every 2 h with a Minolta TIL-1 light meter) and seated in a recliner chair in the CCC as previously published.(20) DLMO was calculated by the "3k" method.(39) Intestinal permeability was measured as 24 hour urinary sucralose represents "primarily" colonic or whole gut permeability as previously described.(40)

Short Chain Fatty Acids

Plasma was collected at 6 times points four hours apart during each phase assessment: (16:00, 20:00, 00:00, 04:00, 08:00, and 12:00) under dim light conditions and constant routine. Gut derived plasma SCFAs – Acetate (Ac), Propionate (Pr), and Butyrate (Bu) we measured by GC-MS assay as previously described.(34)

Rest-Activity Rhythms

All subjects wore a wrist actigraphy monitor (Philips Respironics) for the seven days prior to each phase assessment at 30 s epocs. Actigraphy monitoring allows for assessment of gross motor activity in order to establish defined rest-activity cycles. See Statistical Analysis for further details.

Urinary Melatonin Assessment

Urine was collected for 24 hours for each phase assessment in the CCC. Urinary 6-sulfatoxymelatonin (6-SM), the main metabolite of melatonin, was measured by Bühlmann ELISA kit (ALPCO Diagnostics, Windham, NH) in urine collected in two 12 hour intervals.

Statistical Analysis

Chi square tests and Mann-Whitney *U*-test were used to compare the two subject groups or Analysis of Variance (ANOVA) when comparing three groups. Correlation was done by linear regression. AUC calculated via the trapezoid method. Statistical significance was determined by using a *P* value of <0.05. Statistics were performed using SPSS version 19.0 and rest-activity rhythms were caluculated in R (R v. 3.8.1) with via cosinor or nParACT package.(41) For SCFA analysis, a single cosinor method was used to analyize the data over time as previously described. (42,43) The equation for the cosinor fit is a follows:

$$Y(t) = M + A\cos(2\pi t/\tau + \phi)$$

Where M is the MESOR (Midline Statistic of Rhythm, a rhythm adjusted mean), A is the amplitude (a measure of half the extent of the variation within the cycle), ϕ is the acrophase (a measure of the time of overall highest value), and τ is the period. The fit of the model was the determined by the residuals of the fitted wave as previously described.(44) A 24 hour period was used for all analysis. A Multivariate Wald test were used to test the null hypothesis that the MESOR, amplitude, or acrophase differ by the covariate (alcohol or group). The circular-circular corrleation of DLMO and acrophase were computed on Oriana (Kovach Computing Services, Wales, UK) which uses with Fisher and Lee method.(45)

For actigraphy data, NparACT calculates 3 distinct variables each informing on different aspects of a circadian rhythm: Interdaily stability (IS), Intradaily variability (IV), and Relative Amplitude (RA). IS is the measure of circadian rhythmicity consistency. The strength of the circadian activity from one day to the next, derived from the 24 hour value from a chi-square periodogram, will range from 0 to 1, with a higher value indicating a stronger stability of the rhythm. IV measures disturbances in circadian rhythmicity; ie circadian rhythm fragmentation. Scores range from 0–2 with values approaching 2 representing a stronger degree to which active and resting states of a circadian rhythm are disrupted. RA is a value derived from the averaged values of the 5 least active hours (L5) over the course of experiment and the 10 most active hours (M10) over the course of the experiment. The L5 and M10 values inform on the restfulness of a defined rest period and the degree of activeness during defined periods of activity, respectively. RA values range from 0–1 with values approaching 1 indicating a greater amplitude difference in rest and active periods, thought to be a robust indicator of circadian rhythm health. To validate the viability of actigraphy data in measuring the extent to which a circadian rhythm may be disrupted, the variables derived from this methodology were compared both to patient reported data from sleep logs.

Results

Clinical Variables

A total of 57 subjects were screened for the study l, and 42 consented, and complete the study. The most common reason the subjects did not participate after being screened was they voluntarily withdrew usually due to scheduling conflicts (7), were lost to follow up (4), had a chronic medical disease (2), or could not get a reliable IV line to collect frequent

blood samples over 24 hours (2). There was a statistical difference between the three groups in terms of age and gender with an older and more likely male subject in the AD group (Table 1).

Short Chain Fatty Acids

Acetate, propionate, and butyrate showed a significant diurnal oscillation by cosinor analysis in DW for acetate (F=4.5, p < 0.01), propionate (F= 2.9, p<0.01), Butyrate/Total (F=3.1, p<0.01), and Total SCFA (F=4.6, p<0.01). In the NW group, with central circadian misalignment, SCFA did not have a significant cosinor fit for acetate (F=1.1,p=0.35), propionate (F=0.1,p=0.94), Butyrate/Total (F=1.9, p=0.16), and Total SCFA (1.0, p=0.37). Similar to central circadian misalignment in NW, SCFA in the AD group also did not have a significant fit by cosinor analysis for acetate (F=1.8, p=0.17), propionate (F=1.5. p=0.24), Total SCFA (F=1.3, p=0.28), oor butyrate/Total ratio (F=1.6, p<0.43) (Fig 1).

In the day worker (DW) group, moderate alcohol for one weFek had a significant decrease in the MESOR, amplitude, and acrophase of Total SCFA at 89.5 ± 5.3 vs 82.0 ± 2.4, 15.6 ± 7.4 vs 12.4 ± 4.7, and −0.9 ± 0.5 vs −1.2 ± 0.4; respectively all with p<0.01. There was a similar decrease in Acetate in MESOR, amplitude, and acrophase at 81.8 ± 5.1 vs 74.9 ± 2.3, 15.4 ± 7.1 vs 12.5 ± 4.5, −0.9 ± 0.5 vs −1.2 ± 0.4; respectively all with p<0.01 For propionate and Bu/Total SCFA ratio alcohol only significantly decreased the MESOR. In the night work group (NW) and alcohol use disorder group (AD), 7 days of daily wine consumption did not did not have a significant cosinor fit (Fig 1). Although SCFAs were not quantitatively different at individual time points by group,one week of moderate alcohol also had a significant suppression in total SCFAs as assessed by the area under the curve (AUC) of SCFAs which decreased from 4358 ± 905.56 to 3803.54 ± 707.61 in the DW and NW (p < 0.05, paired analysis).

Correlation of SCFAs with DLMO and Intestinal Permeability

The timing of the central clock (DLMO) had a negative correlation to time of peak expression of SCFAs before and after alcohol in DW (r=−0.30, p<0.05 and r=−0.35, p<0.05), but this correlation with the central and peripheral clocks was decreased in the NW group and lost in the NW group after alcohol consumption for one week. (Fig 2A) A decrease in total SCFA AUC correlated with increased colonic permeability (% excretion of sucralose 13–24 h) r=0.61, p<0.01). (Fig 2B)

Acrophase of SCFAs compared to DLMO

The timing of peak expression of SCFA (acrophase) was compared to the timing of the central clock (DLMO) in both the DW and NW group. In the DW group there was a significant circular-circular correlation at r=−0.3, p<0.05 that was slightly decreased with alcohol, r=−0.26, p<0.05. (Fig 3A&B) In the NW group there was a significant correlation between DLMO and acrophase at r= −0.12, p<0.05, that became non-significant after alcohol. (Fig 3C&D)

Comparison of Circadian Rest-Wake Activity by Wrist Actigraphy

Mean interdaily stability values, prior to alcohol consumption, for the DW, NW and AD cohorts were .55, .29, and .38, respectively. (Fig. 4A). Upon consuming alcohol in the second week of experimentation, the interdaily stability scores dropped in the DW cohort from .55 to .40 (p<.02) and in the NW cohort from .29 to .20 (p<.01) (Fig. 4B). The mean baseline relative amplitude scores for the DW and NW cohorts were .86 and .55, respectively while the AD cohort had a mean relative amplitude value of .67. relative amplitude scores in the NW cohort post-alcohol consumption dropped from .55 to .47 (Fig 4C&D).

Mean intradaily variability scores at baseline were .74, .73, and .76 for the DW, NW and AD cohorts, respectively. Post-alcohol consumption, the intradaily variability scores in the DW cohort shifted from .74 to .73 and in the NW cohort they shifted from .73 to .62 (Fig 4E&F).

Correlation of Circadian Rest-Wake Activity and DLMO

To access the correlation between the rest-wake rhythmicity by the gold standard, DLMO, and wrist actigraphy (Relative amplitude) a circular –linear regression analysis was performed. There was a significant correlation between RA and DLMO in the DW group pre and post alcohol at r=0.77, P<0.05 and r=0.87, p<0.05. (Fig 5A&B) In the NW group the circular-linear correlation between DLMO and relative amplitude was not significant. (Fig 5C&D) The mean DLMO for the AD cohort was 19 h 58 min ± 1 h 18 min. In the AD group, there was a significant correlation between DLMO and relative amplitude at r+0.45, p<0.05). (Fig 5E)

Urinary Melatonin

Urinary 6-SM was measured in each group after a 24-hour urine collection. At baseline, urinary 6-SM was 28.3 ± 15.9, 61.8 ± 32.8, and 79.5 ± 56.4 in the AD, DW, and NW groups respectively, with a significantly lower urinary melatonin value in the AD group (p<0.01). After moderate red wine consumption for 7 days, urinary 6-SM was 29.7 ± 12.5 and 52.4 ± 35.23 in DW and NW, respectively; a significant decrease in urinary 6-SM by paired analysis (p<0.01). Fig 6A and 6B.

Comparison of Urinary Melatonin to Plasma Melatonin AUC

Plasma AUC, showed a significant linear correlation to urinary 6-SM in DW pre and post alcohol, NW pre and post alcohol (P<0.05), and AD subjects. Fig 7.

Discussion

Recently, there has been an increased understanding of how SCFAs may also acts as a zeitgeber in the peripheral tissues,(46) unlike the central circadian clock where the main zeitgeber is light. However, food timing is the zeitgeber for the intestinal epithelial cells, and contributes to the diurnal oscillation of intestinal microbiota and microbiota derived SCFAs. (47) Thus, plasma SCFA should be impacted in circadian misaligned host. Indeed, in the present study we found that: (1) gut derived SCFAs have a diurnal rhythm in humans which is impacted by the central clock of the host; (2) shift work and excessive chronic alcohol

consumption, as in patients with alcohol use disorder, disrupts the oscillation of plasma SCFAs and (3) moderate alcohol consumption for one only week suppresses total SCFAs levels and this effect appears to have important functional impact because it correlates with increased colonic permeability in night shift workers who had disrupted circadian rhythms. Previously we published that central circadian misalignment increased colonic permeability with moderate alcohol.(20) These findings suggest SCFAs as potential mechanism for loss of resiliency on the colonic barrier in a disrupted circadian host.

Microbiota derived SCFAs are produced by fermentation of non-digestible fiber, and are a key component in intestinal barrier homeostasis. For example, butyrate induces epithelial cell proliferation, increases mucin production, is anti-inflammatory, and has protective effects on the epithelial barrier including tight junction proteins (TJPs) integrity.(48),(49) Butyrate enhances intestinal barrier function by facilitating TJPs assembly of ZO-1 and occludin *in vitro*,(49) and can decrease bacterial translocation.(50) Pretreatment with Butyrate, acetate, or propionate prevents TJP disruption and ameliorates alcohol-induced hyperpermeability in a dose dependent manner *in vitro*.(26) Therefore, circadian misalignment and alcohol both appear to impact gut derived SCFAs and colonic barrier homeostasis likely mediated via disruption of TJPs. The impact of central circadian disruption dissociating the diurnal variation of peripheral SCFAs is of particular interest as SCFAs could be a key signal through which the central and peripheral clocks communicate as either a peripheral tissue zeitgetber (46) or the marker of SCFA producing intestinal bacteria.

Previously, we have also published that either environmental or genetic disruption of central circadian clock can decrease resiliency of the intestinal barrier by disruption of TJPs.(51) Gut derived SCFA interact with receptors on the intestinal epithelial cells through G-protein coupled receptors (GPCR) like Gpr41, Gpr43, Gpr109a, and Olfr78. SCFA have been shown to activate signal transduction through GPCR with alcohol to cause intestinal tissue inflammation via effector T cells,(52,53) and impact other key gastrointestinal functions such as diurnal colonic contractility.(54) It is interesting to note that diurnal variation of acetate had higher concentrations during the night, where propionate and butyrate had higher concentrations during the day. (Figure 1). Previously, we reported decreased intestinal barrier resiliency to an alcohol binge during the rest phase in mice,(55) and since propionate and butyrate are more potent ligands for GPR41 and GPR43 than acetate,(56,57) this further suggests disrupted diurnal oscillation of SCFA production by gut microbiota may impact intestinal barrier resiliency. Butyrate or propionate, are also both potent inhibitors of histone deacetylase (HDAC), and an increase in HDAC1 and HDAC2 has been shown to prevent intestinal inflammation and colitis.(53,58) Furthermore, circadian regulation of intestinal microbiota may be mediated through HDAC.(59) Further studies are needed to further examine the mechanisms through which gut derived SCFAs maintain intestinal barrier homeostasis in conjunction with changes in intestinal microbiota to determine their relationship with the timing of central and peripheral circadian rhythms.

A second emphasis of this study was to demonstrate the viability of less invasive, more practical means by which to assess circadian rhythm disruption; i.e. wrist actigraphy and urinary melatonin. We found that: (1) chronic and moderate alcohol disrupt rest-activity

rhythms and suppress urinary melatonin; and (2) rest-activity rhythms and urinary melatonin significantly correlate with the gold standard measure of central circadian rhythms in humans – DLMO. In humans, measurement of the central clock has been extensively studied, with a timing of ~24.2 hours,(60) and the gold standard of measurement is by plasma or salivary melatonin onset under dim light conditions to calculate the dim light melatonin onset (DLMO).(61–64) A secondary marker of circadian misalignment is melatonin amplitude or area under the curve (AUC).(65) Measurement of DLMO is tedious and requires hourly blood or saliva sampling in a controlled laboratory setting for up to 24 hours or longer. This makes DLMO impractical for large-scale human trials and the development of less invasive and at home biomarkers of circadian rhythms are critical. The most widely used biomarker of circadian misalignment is the main metabolite of melatonin, 6-sulfatoxymelatonin (6-SM), which has been used in large scale studies and has been associated with obesity and the onset of diabetes(66,67). Rest-activity patterns by wrist actigraphy have also been used to access central circadian rhythms in cancer treatment, (68,69) and by varying statistical approaches.(41,70) Although urinary 6-SM, overall correlates well with plasma melatonin,(71,72) and rest-activity rhythms in adolescents by wrist actigraphy correlation with salivary melatonin in small groups of subjects (.38 to .82); (73,74) however, comparisons of 6-SM to plasma melatonin and comparison of wrist actigraphy newer statistical methods has not been done.(41) Further establishment of these biomarkers to a gold standard will allow for a better understanding of chronobiology in health and disease.

In the present study, relative amplitude by wrist actigraphy had the best correlation with DLMO in day worker (DW) group, had a significant but less robust correlation in the patients with alcohol use disorder (AD) group, and was significantly correlated in night shift worker (NW) group. Plasma melatonin correlated well with urinary melatonin in all three groups when measured over 24 hours. Alcohol consumption (either chronic alcohol consumption in the AD group or moderate alcohol consumption in the DW and NW group) had a significant disruption in the stability and amplitude of circadian rhythms as measured by 24 hour urinary 6-SM or by rest-activity rhythms as evidenced by moderate alcohol significantly decreasing interdaily stability and relative amplitude but not impacting intradaily variability These finding highlight the feasibility on noninvasive markers of circadian rhythms in human subjects, the significant correlation with a gold standard (DLMO), and the impact of established human behaviors (shift work and alcohol consumption) that disrupt circadian alignment. In short, these noninvasive markers of circadian rhythmicity show great potential in future larger population studies.

There are several limitations to the present study. First, we could not standardize the time of alcohol consumption due to work schedules. However, we aligned time of drinking to sleep/ wake cycle and thus controlled the impact of time of drinking to key circadian output- sleep/ wake cycle. The day workers (DW) drank the wine in the evening after work before they went to sleep; while the night shift workers (NW) drank their wine in the morning after work before they went to sleep or the evening before they went to sleep during the days they did not work. Second, measures of rest-wake activity and urinary melatonin in this study examined patients who were at home in their natural environments during the two weeks of the study, and not done in lab under controlled conditions; however, subjects were asked to

maintain their typical sleep and wake schedule to minimize variability. Third, we could not completely control for the number of night shifts worked each week due to varying work schedules in this population of nurses. We had no significant difference in the number of night shifts worked each week before and after alcohol, but it was not identical in every study subject. Finally, our sample size is limited, due in part to our decision to do more intensive 24 hour in lab phase assessments with plasma melatonin (taken through an indwelling IV), and in this limited sample size we had a significant difference in age and gender between groups. The AD group had more males and was older and the DW or NW group both factors which can affect circadian timing.(79)

In summary, the main findings of this study are: (1) that gut derived SCFAs have a robust diurnal rhythmicity that is disrupted by night shift work and acute or chronic alcohol consumption; (2) loss of resiliency of the colonic barrier is associated with a decrease in SCFAs; and (3) noninvasive makers of circadian rhythms by both rest-wake activity and urinary melatonin correlate well with the gold standard (DLMO), which has exciting potential for future large scale human studies. Further studies are needed to assess the mechanisms through which circadian misalignment and alcohol consumption impact the intestinal microbiota, bacterial products such as SCFAs, and colonic barrier homeostasis. Identifying factors associated with colonic barrier dysfunction and alcohol induced tissue injury could offer not only risk stratification for clinicians but could also lead to the development of novel targets for future investigations.

Acknowledgments:

Abbreviations

SCFAs	short chain fatty acids
AD	alcohol use disorder
DW	day workers
NW	night workers
DLMO	dim light melatonin onset
6-SM	6-sulfatoxymelatonin
SCN	suprachiasmatic nucleus

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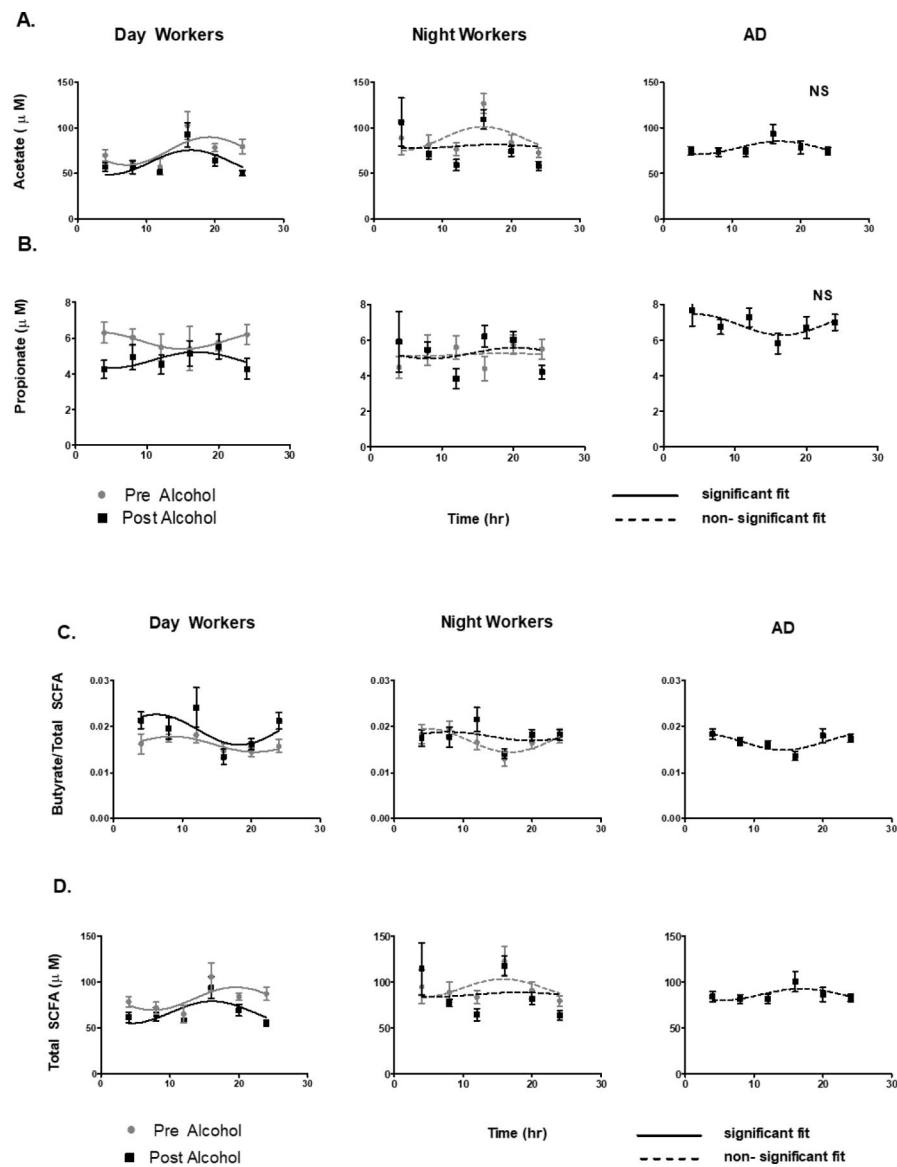
Figure 1: Cosinor Rhythmicity of plasma SCFAs
The cosinor rhythm of plasma SCFAs was test at 6 timepoints over 24 hours under constant routine in all three groups: DW, NW, and AD in **A)** Acetate; **B)** Propionate; **C)** Butyrate/Total SCFAs; and **D)** Total SCFAs. In DW and NW two 24 hour phase assessments were completed before and after 0.5 g/kg of alcohol. A solid line represents a significant 24 hour cosinor curve fit and a dahsed line represents an insignificant 24 hour cosinor curve fit.
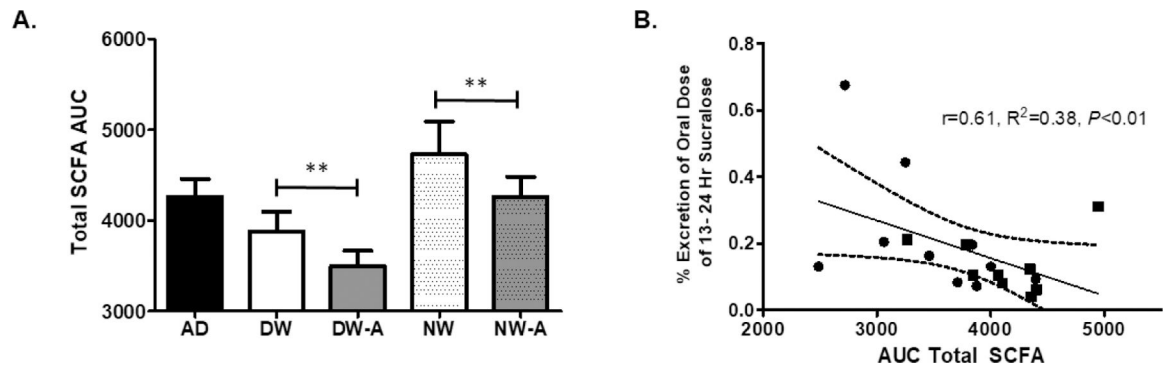
Figure 2: Total SCFA AUC compared to Colonic Permeability

Total SCFA as calculated by area under the curve (AUC) in day workers (DW) and night workers (NW). **A)** Moderate alcohol for one week, 0.5 g/kg, suppressed total SCFAs as measured by Total SCFA area under the curve (AUC) in day workers (DW) and night workers (NW). **B)** A decrease in Total SCFA was correlated with an increase in primarily colonic permeability as measured by urinary sucralose collected 13–24 hours after ingestion. ** denotes a P < 0.05 by Wilcox Signed Rank Test. A linear regression was done to compare Total SCFA to urinary sucralose.
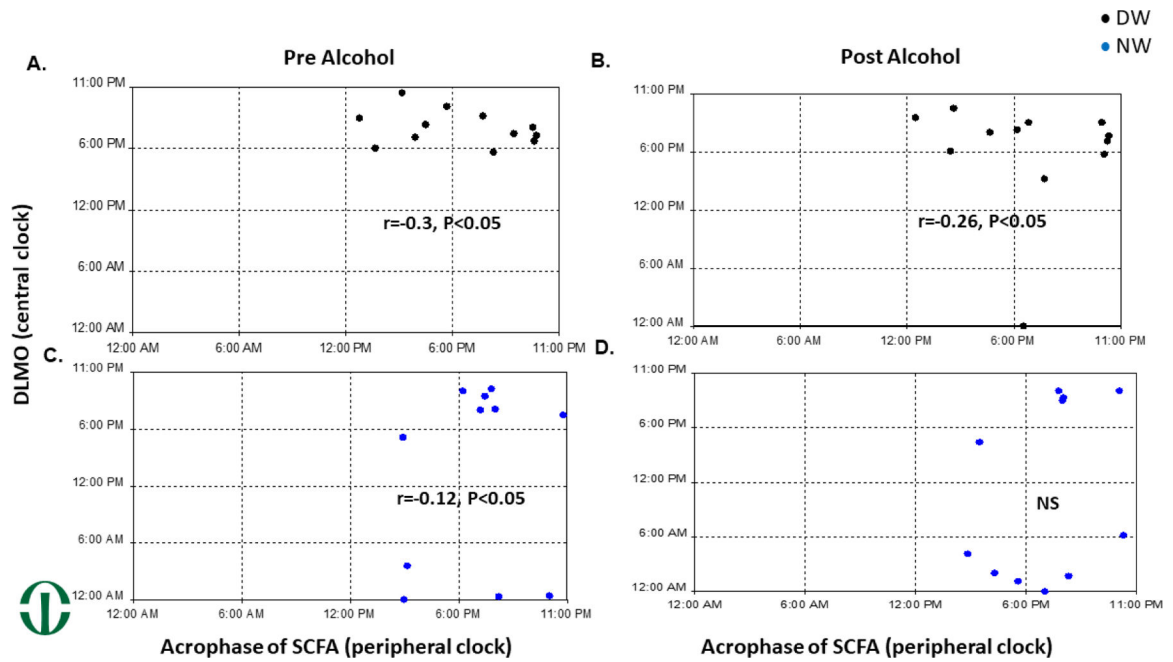
Figure 3: Disrupted circadian host loses synchronization between central and peripheral timing
The time of dim light melatonin onset (DLMO) marker of the central clock was compared to the time of peak expression of SCFAs (the acrophase). By a circular to circular correlation, there was a significant correlation with DLMO and SCFA Acrophase in A) Day Workers (DW) pre alcohol, B) DW post one week of alcohol (0.5g/kg), and C) NW pre alcohol. In NW after one week of alcohol (0.5g/kg) that correlation was not significant.
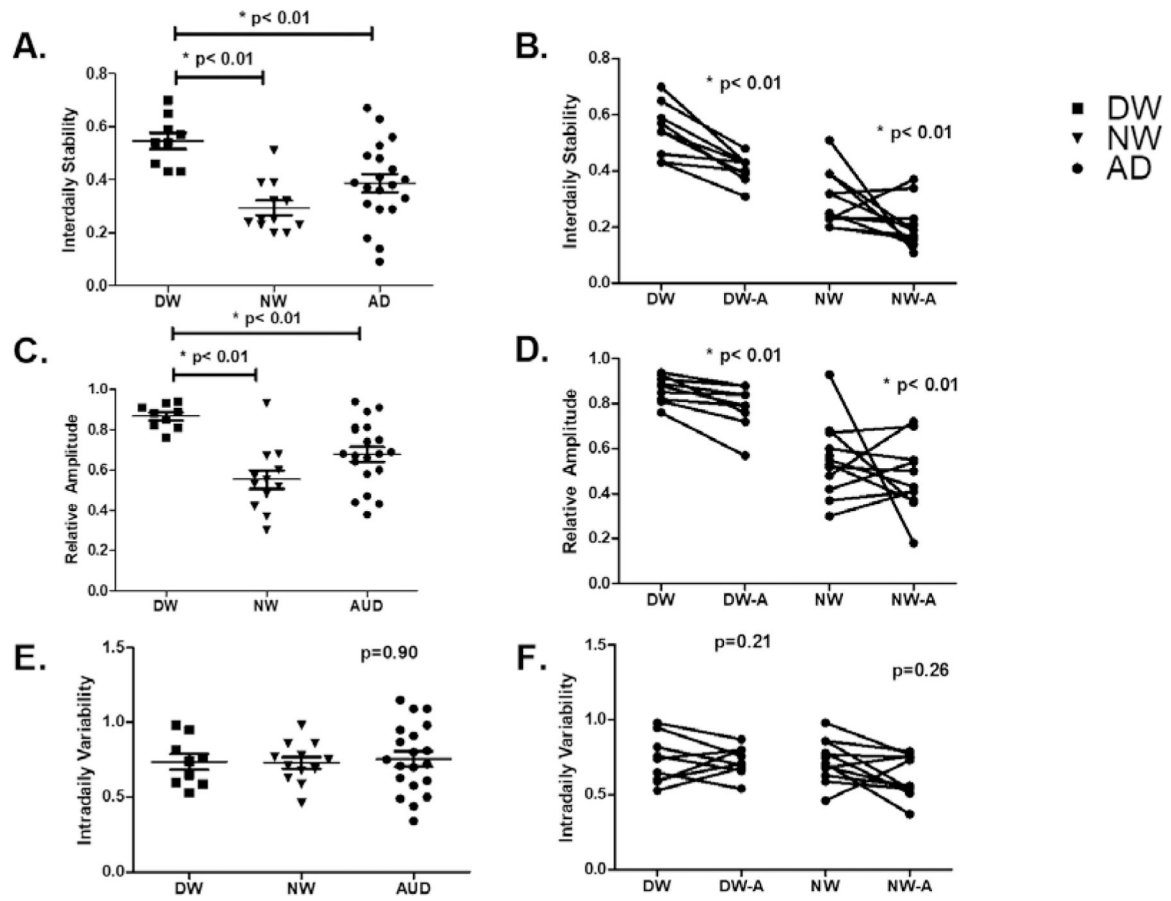
Figure 4: Circadian Rest-Activity Disruption with Alcohol

Non-parametric analysis of rest-activity rhythms for one week prior to each phase assessment in all three groups was used to calculate the interdaily stability (IS), intradaily variability (IV), relative amplitude (RA). **A) and C)** At baseline, there was a significant differenat in IS and RA between DW and both NW and AD (P<0.01). **E)** There was not a significant difference in IV. **B) and D)** After one week of moderate alcohol there as a significant decrease in IS and RA by paired analysis. **F)** There was not a significant change in IV by paired analysis.
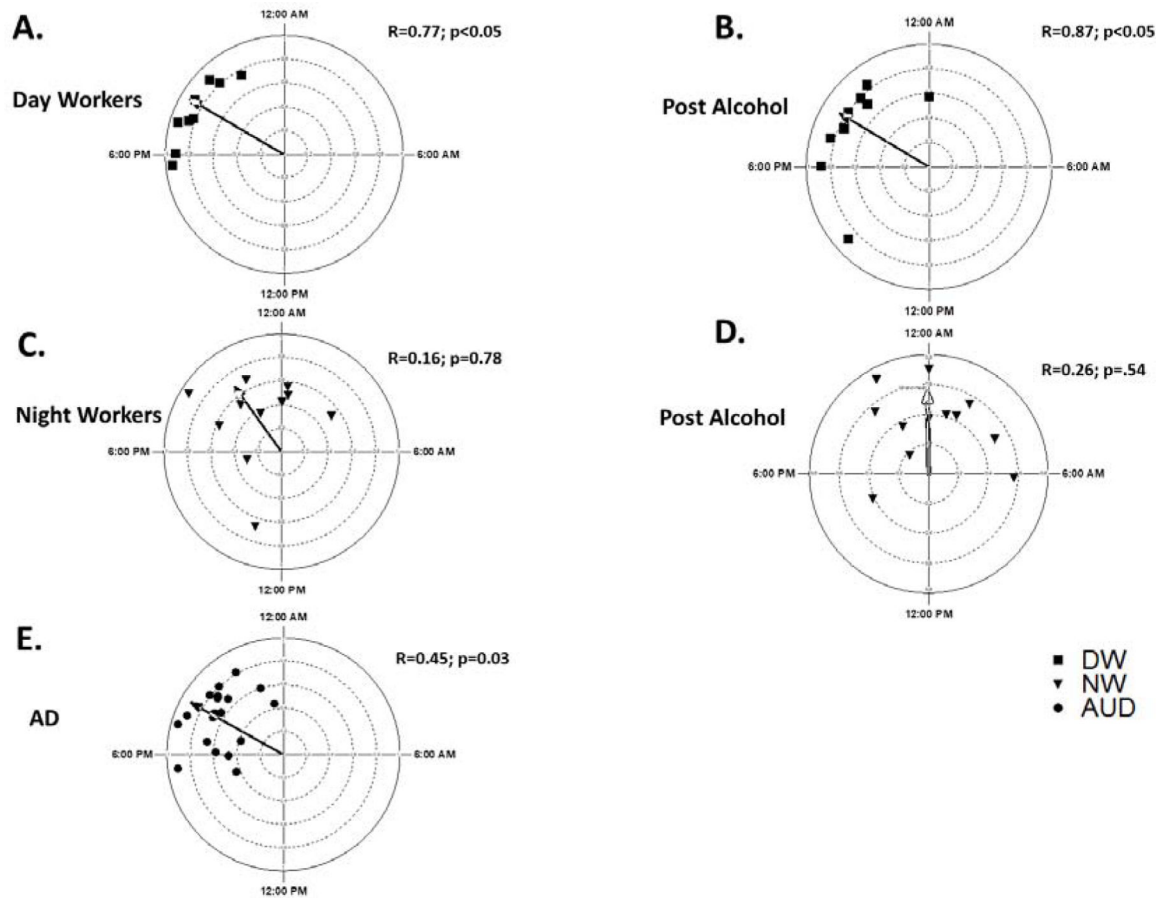
Figure 5: Circadian Rest-Activity Relative Amplitude Correlated to DLMO

Comparing the timing of dim light melatonin onset (DLMO) to circadian rest-activity rhythms by wrist actigraphy there was a significant circular to circular correlation in **A)** Day Workers (DW) pre alcohol and **B)** DW post alcohol. In Night Workers (NW), **C)** and **D)** RA did not correlate with DLMO. In Alcohol use disorder (AD) **E)** there was a less robust but significant correlation between DLMO and RA.
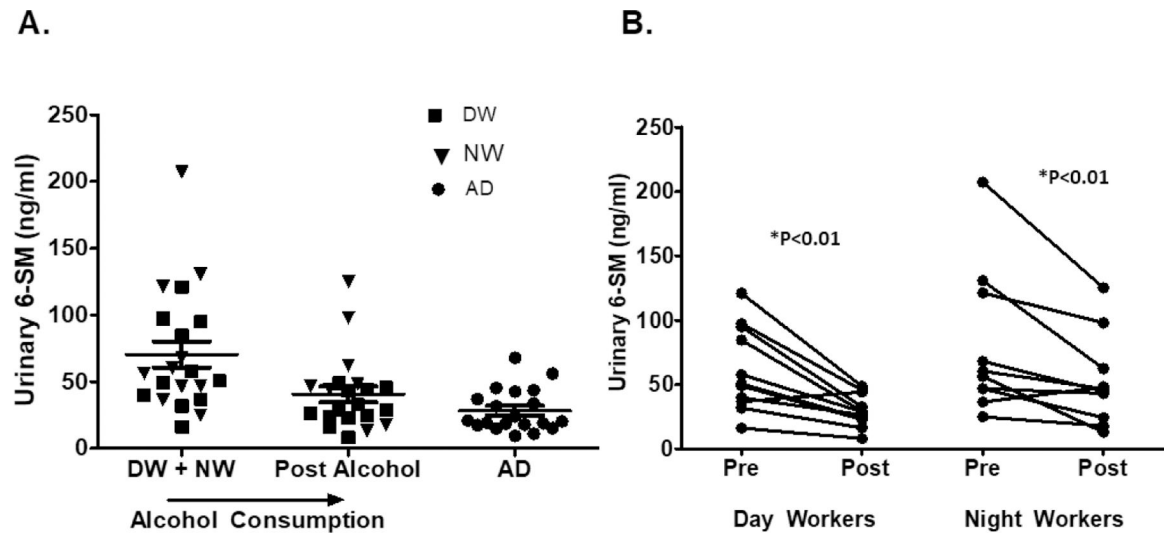
Figure 6: Effect of Alcohol Consumption of Urinary 6-sulfatoxymelatonin
Urinary 6-sulfatoxymelatonin (6-SM) was collected in all 3 groups over 24 hours during each phase assessment. **A)** Moderate alcohol for one week in Day Workers (DW) and Night Workers (NW) caused a significant decrease in and chronic alcohol in the alcohol use disorder (AD) group. **B)** By paired analysis, urinary 6-SM decrease in both Day workers and Night Workers by Wilcox Signed Rank Test.
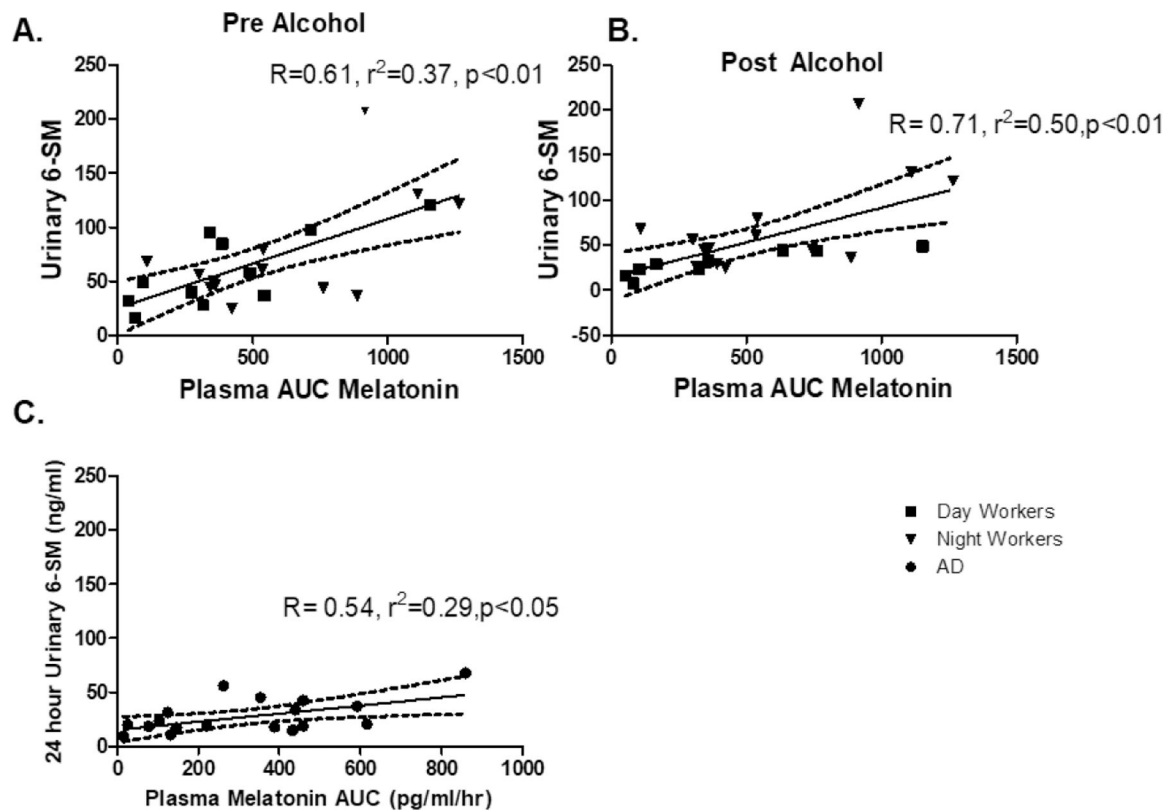
Figure 7: Correlation of Urinary 6-SM and plasma Melatonin AUC

Hourly plasma melatonin taken for 24 hours was compared to a 24 hour collection of urinary 6-sulfatoxymelatonin (6-SM). In all three groups there was a significant correlation with plasma melatonin and urinary 6-SM in A) Day Workers, B) Night Workers, and C) Alcohol Use Disorder (AD). Linear correlation was used to compare plasma and urinary melatonin.

Table 1:

Subject Characteristics

	AUD (N= 20)		DW (N= 11)		NW (N=11)		p-value (<)
	Mean	SD	Mean	SD	Mean	SD	
Age, Mean	45.9	12.2	34.7	11.1	27.7	6.9	0.01
Gender (male n; female n)	15M;5F		10F;1M		11 F		0.01
BMI	28.9	5.6	27.7	7.1	25.0	4.7	0.22
Employment	10U;10E		11 E		11 E		0.01
Years of Heavy Drinking	27.5	11.3	0	0	0	0	0.01
Ethnicity	4C;16AA		10C;1L		10C;1L		0.01
Average Drinks/week	55.0	41.3	0.25	4.3			0.01
Maximum Drinks/day	10.5	4.9	1	0.55			0.01
Days Drink/Month	18.1	8.9	2	4.65			0.01
Adverse Life Events Related to Alcohol; n, %	13, 70%		0		0		0.01
Tobacco Use, n, %	9, 45%		2, 18%		1, 9%		0.01
Illicit Drug Use, n, %	8,40%		0, 0%		0, 0%		0.01