Title

**Histone deacetylase gene expression following binge alcohol
consumption in rats and humans**

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1 **Abstract**

2 **Background**

3 Alcohol binge drinking is one of the most common patterns of excessive alcohol
4 use and recent data would suggest that histone deacetylases (*HDACs*) gene
5 expression profiling could be useful as a biomarker for psychiatric disorders.

6 **Methods**

7 This study aimed to characterize the gene expression patterns of histone
8 deacetylases (*Hdacs 1-11*) in samples of rat peripheral blood, liver, heart,
9 prefrontal cortex and amygdala following repeated binge alcohol consumption
10 and to determine the parallelism of *Hdac* gene expression between rats and
11 humans in peripheral blood. To accomplish this goal, we examined *Hdac* gene
12 expression following 1, 4 or 8 alcohol binges (3 g/kg p.o.) in the rat, in patients
13 who were admitted to the hospital emergency department for acute alcohol
14 intoxication, and in rats trained in daily operant alcohol self-administration.

15 **Results**

16 We primarily found that acute alcohol bingeing reduced gene expression (*Hdac1-*
17 *10*) in the peripheral blood of alcohol-naive rats and that this effect was
18 attenuated following repeated alcohol binges. There was also a reduction of
19 *Hdac* gene expression in the liver (***Hdac2,4,5***), whereas there was increased
20 expression in the heart (*Hdac1,7,8*) and amygdala (*Hdac1,2,5*). Additionally,
21 increased blood alcohol concentrations were measured in rat blood at 1-4 h
22 following repeated alcohol bingeing, and the only group that developed hepatic
23 steatosis (fatty liver) were those animals exposed to 8 alcohol binge events.
24 Finally, both binge consumption of alcohol in humans and daily operant alcohol
25 self-administration in rats increased *Hdac* gene expression in peripheral blood.

1 **Conclusions**

2 Our results suggest that increases in *HDAC* gene expression within the
3 peripheral blood are associated with chronic alcohol consumption, whereas
4 *HDAC* gene expression is reduced following initial exposure to alcohol.

5

6 **Key words:**

7 Histone deacetylases, gene expression, alcohol binge, human and rat,
8 translational research,

9

1 **Introduction**

2 Binge alcohol consumption is one of the most common patterns of excessive
3 alcohol use (CDC, 2014) and can be defined as a drinking pattern that
4 increases the blood alcohol concentration (BAC) by up to 80 mg/dL or more.
5 This generally requires the consumption of at least four or five drinks (for
6 women and men, respectively) within a span of two hours (NIAAA, 2015;
7 Crabbe et al., 2011; Filmore and Jude, 2011). Binge drinking is associated with
8 many immediate consequences, including intentional and non-intentional
9 injuries, death from overdose, and additional long-term consequences if
10 repeated over time (Crabbe et al., 2011). Chronic binge drinking leads to liver
11 and heart disease, abnormal electrophysiological signals within the brain, and
12 psychological impairment (López-Caneda et al., 2013; Maurage et al., 2012;
13 Prada et al., 2012). Therefore, it would be useful if clinicians had the ability to
14 reliably assess the progression of alcoholism through biomarker analysis, which
15 in addition to identifying individuals suffering from alcohol abuse, would further
16 allow the efficacy of pharmacological interventions to be monitored (Litten et al.,
17 2010).

18 Recently, several biomarkers for alcohol-related phenotypes have been
19 identified, many of which are associated with the enzymes utilized during
20 alcohol metabolism and the concentration of their resultant metabolites
21 throughout different bodily tissues and fluids (for reviews see Litten et al., 2010;
22 Bühler et al., 2015). A relatively new approach of detecting novel biomarkers
23 utilizes the analysis of gene expression patterns within the peripheral blood,
24 integrating genomics and environmental influence. Such analyses have been
25 accomplished in studies researching a variety of different neuropsychiatric and

1 neurological diseases, such as Alzheimer's disease, Parkinson's (Booij et al.,
2 2011; Masliah et al., 2013), and very recently schizophrenia and major
3 depressive disorder (Guidotti et al., 2014; Redei et al., 2014). Early preclinical
4 studies evaluating mouse and rat brain tissues revealed that binge consumption
5 of alcohol altered gene expression. For example, it has been demonstrated that
6 the expression of select neurotransmitter receptor genes is decreased following
7 alcohol exposure (Coleman et al., 2011), particularly in cholinergic, GABAergic
8 and peptide genes. Conversely, the expression of genes involved in intracellular
9 signaling pathways in the extended amygdala and prefrontal cortex is increased
10 (McBride et al., 2014; Vetreno and Crews, 2012). While these studies have
11 produced promising results, the alteration of gene expression patterns within
12 the peripheral blood following alcohol bingeing remains poorly characterized,
13 even in the cases of genes that control gene expression (i.e., epigenetic genes).

14 Along with DNA methylation, histone deacetylation is one of the main
15 epigenetic mechanisms that controls gene silencing. In mammals, histone
16 deacetylation is catalyzed by four classes of histone deacetylases (HDACs)
17 (Egger et al., 2004). Eighteen human HDACs have been identified thus far and
18 are grouped into four classes: Class I (comprising HDACs 1, 2, 3 and 8), Class
19 IIA (comprising HDACs 4, 5, 7 and 9), Class IIB (HDACs 6 and 10), Class III
20 (Sirtuins 1-7) and Class IV (HDAC11) (Lombardi et al., 2011). Each HDAC class
21 has a unique bodily tissue distribution. Class I HDACs are ubiquitously
22 distributed; Class II and IV HDACs are located mainly within the heart, skeletal
23 muscle, brain, liver and kidney; and Class III-Sirtuins are expressed either
24 ubiquitously or in a wide range of tissues (Dokmanovic et al., 2007; Nogueiras
25 et al., 2012). Recent studies have shown that *HDAC* gene expression is altered

1 following alcohol exposure both *in vivo* and *in vitro*, although the results are
2 heterogeneous. For example, alcohol has been shown to induce *HDAC1* and
3 *HDAC3* gene expression in human SK-N-MC cells (Agudelo et al., 2012), to
4 inhibit *Hdac1* and *Hdac4* gene expression in a model of rat hippocampal-
5 entorhinal cortex brain slice cultures (Zou et al., 2014), and to decrease *Hdac11*
6 gene expression in mouse striatum tissue (Botia et al., 2012). These results
7 strongly suggest that it would be helpful to conduct translational studies
8 examining *HDAC* gene expression patterns in both humans and animal models
9 following the administration of alcohol to determine the parallelism among
10 species, tissues and treatments.

11 In this context, this study aimed to first characterize *Hdac* gene
12 expression in the peripheral blood, liver, heart, prefrontal cortex and amygdala
13 following repeated alcohol bingeing in the rat. The next aim was to determine the
14 correlation of *Hdac* gene expression among these tissues and finally to examine
15 the parallelism of *Hdac* gene expression between rats and humans in peripheral
16 blood *in vivo*. The brain structures chosen for analysis have been shown to play
17 a predominant role in alcohol related behaviors (Gilpin et al., 2015; Goldstein et
18 al., 2011; Stephens et al., 2008; Vilpoux et al., 2009), while the liver and heart
19 are known to be associated with alcohol-induced injury. Analysis of the
20 peripheral blood was included due to its accessibility for biomarker tests.

21 MATERIAL AND METHODS

22 Animal Studies

23 *Subjects*

24 Forty-eight male Wistar rats were used for the binge-like drinking protocol, and
25 20 were used for the operant alcohol self-administration experiment. These

1 animals were purchased from Harlan Laboratories (Barcelona, Spain), weighed
2 275-325 g at the start of the experiments, and housed in groups of 4 per cage in
3 a temperature- and humidity-controlled environment ($21\pm1^{\circ}\text{C}$) on a 12 h reverse
4 light/dark cycle (lights off at 08:00 h). Binge alcohol treatments and operant self-
5 administration sessions were performed during the dark phase, as rats are
6 nocturnal. Food and water were available *ad libitum* during the experimental
7 procedures except in select conditions as specified below. All research was
8 conducted in strict adherence with the European Directive 2010/63/EU on the
9 protection of animals used for scientific purposes.

10

11 *Binge Alcohol Treatment and Experimental Design*

12 To habituate the animals to intragastric (i.g.) fluid delivery and to reduce
13 nonspecific stress responses prior to alcohol administration, rats were
14 administered tap water (i.g.) once per week for two weeks. The animals were
15 then assigned into one of the following groups: the control group (administered
16 water) and the alcohol binge groups, comprising 1 alcohol binge, 4 alcohol
17 binges, and 8 alcohol binges (see **Figure 1**). Binge alcohol treatment was
18 performed once per week on Thursdays. Alcohol was orally administered
19 through gavage (i.g.) at a dose of 3 g/kg using a 25% alcohol solution in tap
20 water at a volume of 15 mL/kg. We used this dose of alcohol to obtain
21 comparable blood alcohol concentrations to those found in humans after alcohol
22 binge (approximately 240 mg/dL from our human samples). Control animals
23 received the same volume of liquid as above but were given tap water alone.
24 The rats were deprived of food for 12 h prior to the i.g. treatment to normalize

1 the absorption of alcohol among the animals (avoiding the presence or absence
2 of food into their stomach).

3 Blood samples were collected for analysis of blood alcohol
4 concentrations from the tail caudal vein at 60, 120, 180 and 240 min following
5 alcohol binge (see Figure 1). To avoid cutting the rat tail four times, half of the
6 animals (i.e., n=6) were used to collect the blood at 60 and 180 min and the
7 other half at 120 and 240 min. In the eighth week, post binge blood samples
8 were collected only at the 120 min time point, as all animals were then
9 sacrificed following blood collection. The other experimental groups were
10 treated in the same way every week, and the blood samples of the group that
11 was administered water only were used as a control. In the eighth week, for the
12 analysis of gene expression, all animal cohorts were sacrificed by decapitation
13 at 120 min following binge alcohol treatment, and the blood, liver, heart and
14 brain (prefrontal and amygdala) tissues were immediately dissected on ice and
15 frozen on dry ice at -80 °C. Frozen tissue samples were stored for subsequent
16 histology and gene expression analysis.

17

18 *Operant Alcohol Self-administration*

19 To habituate rats to operant alcohol self-administration, we followed the protocol
20 described by Echeverry-Alzate et al. (Echeverry-Alzate et al., 2014). Briefly,
21 operant alcohol sessions were conducted in twenty modular chambers (Med
22 Associates Inc., St. Albans, VT, USA). The chambers were equipped with two
23 retractable levers on either side of a drinking reservoir positioned in the center
24 of the front panel of the chamber. The levers were counterbalanced to respond
25 as the active lever (delivering 0.1 mL either 10% alcohol v/v or 0.02% saccharin

1 w/v) or as the inactive lever. For the entirety of the study, each operant alcohol
2 session lasted 30 min under a fixed ratio 1 schedule for seven days a week.

3 To analyze gene expression in the animal cohorts described above,
4 peripheral blood samples were collected from the tail vein at 60 min before and
5 60 min after the alcohol self-administration session on the fourth day of 10%
6 alcohol availability. An additional cohort of rats (n=10) only had access to
7 saccharin (0.02% saccharin) and did not consume any alcohol during the study.
8 This cohort served as a control group for assay calibration purposes in PCR
9 experiments.

11 *Alcohol Analysis*

12 To determine blood alcohol concentrations following binge alcohol treatment,
13 250 μ L of blood was collected from the rat tail vein at 60, 120, 180 and 240 min
14 post treatment using a capillary tube (Microvette CB 300 K2E) that contained
15 EDTA dipotassium salt. Whole blood samples were centrifuged for 15 minutes
16 at 1,500 x g using a refrigerated centrifuge, and the plasma was stored at -80
17 °C for further use. The alcohol concentration was measured using the
18 EnzyChrom alcohol assay kit following the protocol recommended by the
19 manufacturer (Bioassay Systems, Hayward, CA, USA). All measurements were
20 performed in duplicate.

22 *Histological study of the liver*

23 As stated above, animals were sacrificed by rapid decapitation, after which
24 livers were immediately harvested and frozen. Each liver sample was divided in
25 half to provide samples for both histology and gene expression analysis. Liver

1 samples were cryosectioned using a LEICA CM3050 cryostat to produce 10- μ m
2 thick sections and stained with Oil Red O to visualize neutral lipid content
3 according to the recommendations of Koopman et al (Koopman et al., 2001). An
4 Oil Red O stock solution consisting of 0.5 g Oil Red O powder and 100 ml of
5 60% triethyl-phosphate was prepared. Oil red O was obtained from Sigma-
6 Aldrich (Steinheim, Germany). Liver sections were fixed in 4% formaldehyde
7 (Merck, Darmstadt, Germany) for one hour at room temperature. During this
8 time and prior to staining, a 36% triethyl-phosphate working solution containing
9 12 mL oil red O stock solution and 8 mL deionized water was prepared and
10 filtered. Following fixation in paraformaldehyde, slides were rinsed three times
11 in water to remove excess paraformaldehyde and then immersed in filtered Oil
12 Red O solution for 30 minutes. Immediately thereafter, slides were rinsed with
13 running tap water and mounted in glycerine jelly. Prior to being mounted, a subset
14 of sections was counterstained using Gills haematoxylin.

15 Lipid droplets were quantified using high-resolution digital
16 microphotographs that were taken with the 40x objective of a Zeiss Axiophot
17 microscope (Oberkochen, Germany) under equivalent conditions of light and
18 brightness and contrast. Densitometry measurements of select regions of each
19 image were obtained for quantification purposes using the analysis software
20 ImageJ 1.383 (NIH, USA). Three separate areas surrounding the central vein
21 from four random tissue sections per animal were evaluated. Analysis included
22 conversion of the color image into a binary image and subsequent
23 measurement of the optical density (OD) from each of three different areas of
24 equal size. The basal optical density was obtained from the average of three

1 separate OD values, which were then averaged for each section. Four sections
2 were averaged for each animal. Six to eight animals were evaluated per group.

3

4 *Real time quantitative PCR experiments for rats*

5 Real time quantitative PCR, which has been described as one of the most
6 powerful tools to quantify gene expression (Schmittgen and Livak, 2008), was
7 performed using a LightCycler 480-II machine (Roche, Barcelona, Spain) with
8 SYBR Green Real-time qPCR master mix (Applied Biosystems, Warrington,
9 UK) and specific primers kept at a concentration of 200-nM (see *Supplemental*
10 *Table 1* for rat primers). The melting curve analyses showed only a single clear
11 peak, and the size of the PCR products were confirmed by agarose gel
12 electrophoresis. A 10-fold dilution series of the template was used to amplify
13 each gene to validate the efficiency of each assay and to confirm that the
14 amplification efficiencies of the target and reference genes were comparable
15 (indicated by a near-zero slope value for both the target and reference genes).
16 The 18S ribosomal RNA gene (18S) was used as an internal control for
17 normalization. The control group of rats that were administered only water was
18 used to calibrate the assay for analysis of the cohort of rats that underwent
19 binge alcohol consumption, whereas the cohort of rats that drank only saccharin
20 was used to calibrate the assay for the cohort that was offered operant self-
21 administration of alcohol. The $2\Delta\Delta CT$ method was used to analyze the
22 expression data (Schmittgen and Livak, 2008). Total RNA was isolated from
23 brain, liver and heart samples using Tripure Isolation Reagent (Roche) and was
24 stored at -80°C . Total RNA was isolated from whole blood using Trizol LS
25 Reagent (Life Technologies, Carlsbad, USA). One microgram of total RNA was

1 reverse transcribed into cDNA using the Transcriptor First Strand cDNA
2 Synthesis Kit (Roche).

3

4 **Human Study**

5 *Subjects*

6 Our clinical study was performed on 20 patients who were admitted to the
7 Emergency Department of the University Hospital of Salamanca (Spain) with
8 moderate to severe acute alcohol intoxication. Acute alcohol intoxication was
9 defined by clinical signs (e.g., slurred speech, ataxia, impaired reasoning,
10 confusion or disorientation), blood alcohol levels > 1 g/L, and the consumption
11 of at least five standard drinks (50 g) in men or four (40 g) in women during the
12 six hours prior to hospital admission. Toxicological urinary analysis was also
13 performed, and patients were excluded from our study if either clinical data or
14 patient history demonstrated the consumption of illegal drugs (apart from
15 cannabis). Additional exclusionary criteria included chronic or acute illness
16 (aside from symptoms produced by acute alcohol intoxication) and the use of
17 medication. The clinical, epidemiological and analytical characteristics of our
18 patient cohort are shown in Table 1. Twenty-two healthy controls (8 male and
19 14 female) with an average age of 23.2 years (SEM±0.45) were also included in
20 the study. Controls were carefully selected to include subjects who did not
21 consume alcohol apart from light sporadic drinking and who reported no alcohol
22 consumption during the 72 hours leading up to the study and no binge drinking
23 episodes during the three months prior. These patients were subject to the
24 same exclusionary criteria as those who binged on alcohol and had normal
25 hematological and plasma biochemical parameters. This study was approved

1 by the Ethics Committee of the University Hospital of Salamanca and written
2 informed consent was obtained from each participant. Blood samples were
3 obtained from patients upon admission for use in standard care as well as for
4 research purposes, and the samples analyzed in this study were only used after
5 patients could understand the details of the study adequately enough to provide
6 informed consent for inclusion.

8 *Real time quantitative PCR analysis of human blood samples*

9 Whole blood samples (2.5 mL) from each individual were drawn into PAXgene
10 Blood RNA tubes (Qiagen, California, USA). Samples were kept at room
11 temperature for at least two hours following collection, and stored for long-term
12 access at -20 °C. Total RNA was isolated from each sample using the PAXgene
13 Blood miRNA Kit (Qiagen, Valencia, CA) according to manufacturer's
14 instructions, and processed samples were stored at -80 °C. The concentration
15 and purity of extracted RNA samples were assessed using a Nanodrop ND-
16 1000 spectrophotometer. Further processing of RNA samples was identical to
17 that described in the animal PCR study section described above, except for the
18 use of human-specific primer sets (see *Supplemental Table 2* for human
19 primers).

20 **Statistical Analysis**

21 SPSS statistical software (version 20.0) for Windows (Chicago, IL, USA) was
22 used to perform all statistical analysis. Significant ANOVA results were followed
23 by Tukey's post-hoc tests. The data obtained from experiment 1 were analyzed
24 using one-way ANOVA within each tissue (Figures 2-4), and correlations were
25 determined by Pearson's correlations analysis (**Figure 5**). Data from experiment

1 2 were analyzed using a two-way mixed ANOVA (week x hour) (Figure 6) and a
2 two-sided independent Student's t-test (Figure 7-E). Data from experiment 3
3 were analyzed using a two-way ANOVA (gene x treatment) (Figures 8 and 9).
4 After confirming the significance of our primary findings using ANOVA, a
5 significance level of $p<0.05$ was applied to all remaining statistical analyses.

6

7 **RESULTS**

8 Experiment 1. *Hdac* gene expression in blood, liver, heart, prefrontal cortex and
9 amygdala following repeated binge alcohol consumption in rats

10 The objectives of this experiment were two-fold. The first was to quantify the
11 effect of repeated binge alcohol consumption (3 g/kg) on the gene expression of
12 Class I, IIA, IIB and IV *Hdacs* in samples taken from peripheral blood, liver,
13 heart, prefrontal cortex and amygdala tissues. The second was to assess the
14 potential correlative relationships of *Hdac* gene expression among these
15 tissues.

16 As shown in Figures 2, 3 and 4, which depict the relative fold changes in
17 *Hdac* expression, results varied according to tissue type and to the number of
18 alcohol binge episodes experienced by the subject. One of the more consistent
19 results to emerge from this experiment was the observation that, except for
20 Class IV (*Hdac11*), there was a reduction of *Hdac* gene expression in peripheral
21 blood collected from the tail vein following the initial alcohol binge (e.g., Class I
22 *Hdacs*: one-way ANOVA blood *Hdac1*, $F_{(3,34)}=3.22$, $p<0.05$; *Hdac2*, $F_{(3,32)}=8.09$,
23 $p<0.01$; *Hdac3*, $F_{(3,34)}=4.91$, $p<0.01$; *Hdac8*, $F_{(3,33)}=3.62$, $p<0.05$). With the
24 exception of *Hdac4* and 5 (Figure 3), this reduction in gene expression was
25 generally lost following successive alcohol binge episodes. Additional significant

1 results obtained from these assays include a reduction of *Hdac* gene
2 expression in the liver (*Hdac2*, 4, and 5) and an increase in *Hdac* expression in
3 the heart (*Hdac1*, 7, and 8) and amygdala (*Hdac1*, 2, and 5). Also, *Hdac8*
4 increased its expression in the amygdala but only after eight alcohol binges.

5 We next evaluated whether *Hdac* gene expression patterns among
6 varying tissues correlated to the details of binge alcohol consumption. To
7 investigate this question, we utilized 2^{-C_T} values according to the protocol
8 described by Schmittgen and Livak (2008). Figure 5 shows the corresponding
9 matrix of correlations, in which only statistically significant results ($p<0.05$) are
10 presented. We generally observed that the most frequent positive correlative
11 association was among values obtained from amygdala and heart tissue
12 samples, regardless of the conditions of binge alcohol consumption (e.g.,
13 Pearson correlations from the control group, *Hdac1* $r=0.65$, $p<0.05$; *Hdac2*
14 $r=0.79$, $p<0.01$; *Hdac3* $r=0.81$, $p<0.01$; *Hdac4* $r=0.70$, $p<0.05$). Additionally, we
15 detected that the most frequent negative association was between values
16 measured from prefrontal cortex and amygdala tissue samples (e.g., Pearson
17 correlations 8-alcohol-binges group, *Hdac4* $r=0.75$, $p<0.05$; *Hdac7* $r=0.74$,
18 $p<0.05$). Another finding was that while the highest number of positive
19 correlations was produced within the control group, the highest number of
20 negative correlations came from the cohort exposed to 8 alcohol binges. These
21 observations were in spite of the fact that there was no observed linear
22 relationship between alcohol binge consumption and *Hdac* gene expression
23 across different tissues.

Experiment 2. Determination of blood alcohol levels and histological analysis of the rat liver

The purpose of this experiment was to determine the blood alcohol levels of rats following repeated binge alcohol consumption and to explore the extent of hepatic steatosis (fatty liver) within these animals. Figure 6 shows that blood alcohol concentration peaked during the first hour following consumption and decreased progressively thereafter. Additionally, chronic binge drinking led to increased blood alcohol levels independent of the time point under evaluation (two-way mixed ANOVA: week $F_{(6,108)}=13.27$, $p<0.001$; hour $F_{(3,18)}=15.46$, $p<0.001$; interaction $F_{(18,108)}=0.60$, NS).

The effects that 8 alcohol binge episodes produced on the liver are shown in Figure 7. Steatosis (fat deposition) was observed in hepatocytes. Panels A-D depict representative images stained with either Red Oil O or Red Oil O and Hematoxylin, and Panel E provides the optical density quantification values of the lipid droplets from Oil Red O staining ($p<0.005$, two-sided Student's t-test). No effects were observed in animal cohorts exposed to either 1 or 4 alcohol binges (data not shown).

Experiment 3. Comparison of HDAC gene expression profiles from peripheral blood samples collected from human and rat subjects

The third experiment was designed to explore if there was a parallelism between rat and human HDAC gene expression in peripheral blood following binge alcohol consumption. In contrast to the acute decrease of *Hdac* gene expression observed in rat tissue samples, human tissue samples demonstrated an increase in *Hdac* expression (except for HDAC9 and 10), as

1 shown in Figure 8. Since there were no significant differences between sexes in
2 *HDAC* gene expression the data were analyzed collectively. ANOVA analysis
3 revealed that average *HDAC* gene expression was significantly higher in
4 subjects that were binge drinking alcohol versus the control group and that
5 there were no differences among *HDAC* species or their interactions (two-way
6 ANOVA: treatment $F_{(1,440)}=80.29$, $p<0.001$; gene $F_{(10,440)}=1.45$, NS; interaction
7 $F_{(10,440)}=1.46$, NS). Based on the above results, we conducted a complementary
8 study using operant alcohol self-administration in rats. This allowed us to
9 determine whether *Hdac* gene expression is linked to repeated exposure to
10 alcohol rather than merely to alcohol binging. Figure 9 shows that repeated
11 operant alcohol self-administration led to a general increase in *Hdac* gene
12 expression, which mimicked more closely the increase observed in human
13 samples. This effect was observed at time points both 60 min before and/or 60
14 min after alcohol self-administration (*Before*: two-way ANOVA: treatment
15 $F_{(1,197)}=17.18$, $p<0.001$; gene $F_{(10,197)}=0.69$, NS; interaction $F_{(10,197)}=0.68$,
16 NS)/(*After*: two-way ANOVA: treatment $F_{(1,185)}=31.08$, $p<0.001$; gene
17 $F_{(10,185)}=0.75$, NS; interaction $F_{(10,185)}=0.74$, NS).

18

19 DISCUSSION

20 The aim of this work was to characterize *Hdac* gene expression in a sampling of
21 tissues and the correlations of such in an animal model of binge alcohol
22 consumption and to investigate whether these results could be replicated in
23 humans. Our hope was that this study would facilitate the use of *Hdac* gene
24 expression as a potential biomarker for alcohol abuse and alcohol-induced
25 bodily damage.

Our first major finding was that acute alcohol binge led to a reduction of *Hdac* gene expression in rat peripheral blood and that this response became attenuated following repeated alcohol binges. The obtained values in *HDAC* gene expression in rat tissues were in agreement with previous studies (i.e., Finegersh and Homanics, 2014; Sarkar et al., 2014). As tissue samples were collected at the early time point of just two hours following the alcohol binge, it is clear that changes to *Hdac* gene expression occur rapidly and can be reliably detected using our methodology. These results are in agreement with previous studies. For example, Kirpich et al., (2012) have reported the down-regulation of select *Hdacs* in the mouse liver as early as four hours after three consecutive alcohol binges. Furthermore, Botia et al., (2012) demonstrated a reduction in the expression of *Hdac11* in the mouse striatum as early as 30 min after acute alcohol exposure. Even some studies have repeatedly proven that the activity of HDACs is reduced in the amygdala one hour after alcohol exposure (Pandey et al., 2008; Sakharkar et al., 2014; Pandey et al., 2015), including in selectively bred alcohol-preferring rats (Moonat et al., 2013). Nevertheless, our results show that *Hdac* gene expression in rat peripheral blood undergoes degrees of tolerance as a consequence of repeated alcohol binges. For example, statistically significant differences disappeared in the expression of Class I and IIB *Hdacs*, whereas values measured for Class IIA gene expression (*Hdac4* and 5) remained consistent. Additionally, a reduction of *Hdac* gene expression (*Hdac4* and 5) was observed in the rat liver, while significant increases in gene expression (*Hdac1* and 2) were found in the heart and amygdala following alcohol binge. No significant differences were noted in the prefrontal cortex. The discrepancy of *Hdac* gene expression among varying tissue types might be

1 explained by the fact that different Hdac variants possess different patterns of
2 tissue distribution. For example, class I *Hdacs* are ubiquitously distributed
3 throughout the body, whereas Class IIA and IV *Hdacs* are found within the
4 heart, brain and skeletal muscle. Class IIB *Hdacs* primarily localize to the
5 cytoplasm of liver and kidney cells (Dokmanovic et al., 2007). *Hdac11*, a Class
6 IV Hdac, was the only gene included in our study whose expression was not
7 reduced within the peripheral blood following acute alcohol bingeing. This was
8 likely due to the comparatively low concentration of mRNA measured for this
9 Hdac in rat blood. For example, as a rough estimation based on PCR
10 amplification data obtained from the control group, *Hdac11* was 1.024-fold and
11 128-fold less expressed in peripheral blood than *Hdac7* and *Hdac10*,
12 respectively.

13 We hypothesized that there would be significant correlations in *Hdac*
14 gene expression patterns between the peripheral blood and other examined
15 tissues and that these correlations would be modulated by the number of
16 alcohol binge episodes to which a given subject was exposed; however, this
17 hypothesis was not verified. Instead, we found that the more frequent positive
18 correlation was between the amygdala and the heart, the more frequent
19 negative correlation between the prefrontal cortex and the amygdala; further,
20 the highest number of negative correlations was within the cohort exposed to 8
21 alcohol binges. This last finding indicates that as the number of alcohol binge
22 episodes increases, *Hdac* gene expression becomes more variable among
23 different tissues (i.e., increases in gene expression were observed in select
24 types of tissue, while decreases in gene expression were observed in others).

1 We examined blood alcohol concentrations, as well as alcohol-induced
2 fatty liver (steatosis). As was expected, a reduction in blood alcohol
3 concentration was observed as sample collection was delayed from 1 to 4 h.
4 However, as each alcohol binge treatment occurred only once per week, the
5 significant increases in blood alcohol concentrations following repeated,
6 intermittent alcohol binging was unexpected. We would expect the total
7 clearance of alcohol from the rat in between alcohol binge treatments. It is
8 notable, however, that other authors have reported similar results. For instance,
9 Maier et al., (1995) administered sufficient alcohol to rats (5 g/kg) to result in a
10 state of daily intoxication, and found that the peak of averaged blood alcohol
11 concentrations was higher on the final day of alcohol treatment (18th day).
12 Future studies should examine the biochemical basis for the cumulative nature
13 of blood alcohol concentration following repeated and intermittent alcohol
14 administration. On the other hand, hepatic steatosis only developed in animals
15 subjected to 8 episodes of alcohol binging, despite the fact that there was no
16 direct relationship between alcohol consumption and *Hdac* gene expression in
17 the liver for this cohort and that the liver was the second tissue in which we
18 found a reduction in gene expression. These findings contrast those published
19 by Kirpich et al. (Kirpich et al., 2012, 2013) in which a down-regulation of
20 hepatic *Hdac 1, 7, 9, 10, 11* and an up-regulation of *Hdac3* were found to be
21 directly associated with alcohol binge treatment. One possible explanation for
22 this discrepancy is the use of a markedly different protocol, which essentially
23 focused on alcohol-induced hepatic steatosis. Furthermore, in contrast with our
24 dosing schedule of 3 g/kg administered once per week, Kirpich et al.
25 administered alcohol at a dose of 4.5 g/kg three times, at 12-hour intervals.

1 While this dosing schedule agrees with the recent review of Mathews et al.
2 (2014) regarding animal models of alcoholic liver injury, which claims that the
3 most commonly used doses for the study of alcohol-induced liver injury are 4-6
4 g/kg, we instead chose to use a dose of 3 g/kg to obtain comparable blood
5 alcohol concentrations to those found in humans following the binge alcohol
6 consumption (239 ± 0.13 mg/dL from our human samples).

7 To the best of our knowledge, this is the first study to assess *HDAC* gene
8 expression in human peripheral blood following an alcohol binge. The obtained
9 values in *HDAC* gene expression in human blood were in agreement with
10 previous reports (i.e., Hobara et al., 2010). Overall, we found *HDAC* expression
11 to be increased in response to alcohol, with the exception of the Class IIA
12 *HDAC9* and the Class IIB *HDAC10*. At the present time there are only two
13 published studies that have evaluated the effects of alcohol on *HDAC* gene
14 expression in humans. Both of these also found increased *HDAC* expression,
15 though it should be noted that these studies were performed *in vitro* (Agudelo et
16 al., 2011, 2012). The increase of *HDAC* gene expression that was measured in
17 human peripheral blood samples following an acute alcohol binge contrasts with
18 the reduction of gene expression observed in rat peripheral blood under the
19 same conditions. Our first hypothesis to explain this discrepancy centered
20 around the acute nature of the alcohol binge: in the case of an alcohol-naive rat,
21 the first binge actually represented the animal's first exposure to high quantities
22 of alcohol, whereas the majority of patients presenting with acute alcohol
23 intoxication reported regular alcohol consumption during the weekend.
24 Considering the high rate of underreporting of alcohol use (Boniface et al.,
25 2014), it is very likely that these patients engaged in repeated episodes of binge

1 drinking in the past. To empirically verify our hypothesis, we performed an
2 additional experiment in which rats were allowed operant alcohol self-
3 administration, wherein the animals drank low to moderate doses of alcohol
4 daily and reached blood alcohol levels between 10-40 mg/dL (approximately 25-
5 60 responses were recorded). In peripheral blood samples obtained from this
6 cohort of rats, we found that the expression of select *Hdacs* increased more
7 similarly to what was observed for humans. Such increases in *Hdac* expression
8 were slightly more pronounced 60 min after alcohol self-administration versus
9 60 min before. Collectively, these data suggest that variability in *HDAC* gene
10 expression in response to alcohol exposure is linked to past alcohol use. For
11 example, while initial exposure to alcohol leads to an acute decrease of *HDAC*
12 gene expression in peripheral blood, repeated exposure to alcohol (either in
13 binge form or moderate intake) either does not affect or increases *HDAC* gene
14 expression. A limitation of this operant self-administration study was that the
15 saccharin group was used as the control-group (calibrator) as far as the animals
16 do not work to obtain water.

17 Several researchers of psychiatric conditions have proposed the
18 monitoring of *HDAC* gene expression within human peripheral blood to serve as
19 a biomarker of alcohol-related behaviors. For example, Hobara et al., (Hobara
20 et al., 2010) found that variability in the expression of the same 11 *HDACs* that
21 were investigated here (*HDAC1-11*) was associated with the pathophysiology of
22 mood disorders (essentially a decrease in gene expression was indicative of a
23 remissive state of depression). Taken together, these results indicate that
24 *HDAC* gene expression profiling of peripheral blood may serve as a potential
25 biomarker for many psychiatric conditions. Here, we have performed a set of

translational experiments that provide valuable insights into how *HDAC* gene expression changes in response to alcohol exposure in rats and humans and provide methodology that allows the reliable detection of these changes.

Conflict of Interest

None

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1 **Figure Legends**

2 **Figure 1.** Schematic representation of the experimental design. Red dots
3 represent blood collected following cuts made to the tail veins of rats. White and
4 blue rectangles represent cohorts of animals that were administered either
5 water or alcohol and sham cut. Animal sacrifice was performed at two hours
6 post alcohol binge to increase similarity in alcohol blood concentrations
7 measured between rat and human subjects.

8
9 **Figure 2.** Effects of one, four or eight alcohol binges on **Class I** histone
10 deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart,
11 prefrontal cortex and amygdala. Data represent the mean $\pm$ SEM (n=10-12
12 animals per group) and the relative fold change obtained using the $2\Delta Ct$
13 method (see Methods section). *p<0.05 compared with the control group (no
14 alcohol binge).

15
16 **Figure 3.** Effects of one, four or eight alcohol binges on **Class IIA** histone
17 deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart,
18 prefrontal cortex and amygdala. Please see Figure 2 Legend for details.
19 *p<0.05 compared with the control group (no alcohol binge).

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21 **Figure 4.** Effects of one, four or eight alcohol binges on **Class IIB and IV**
22 histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart,
23 prefrontal cortex and amygdala. Please see Figure 2 Legend for details.
24 *p<0.05 compared with the control group (no alcohol binge).

25

Figure 5. Correlations among histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart, prefrontal cortex (PFC) and amygdala (AMG) following variable alcohol binge conditions. Only correlations with significance lower than $p < 0.05$ are shown (green represents positive correlations, red represents negative; $n = 10-12$ animals per group).

Figure 6. Blood alcohol concentrations over a four-hour period following a weekly alcohol binge (3 g/kg) in rats. In the eighth week, blood samples were only collected at the 120 min time point following binge alcohol treatment, as animals were sacrificed at this time point. Values represent the mean of blood alcohol concentrations $\pm$ SEM ($n = 6$ animals per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the first hour. # $p < 0.001$ compared with the first week (first alcohol binge).

Figure 7. Upper panels depict 20x representative microphotographs of Oil Red O-Hematoxylin stained liver sections obtained from the control group of rats (A), and the group exposed to 8 alcohol binges (B). Lower panels depict Oil red O staining of lipid droplets of the control group (C), the group exposed to 8 alcohol binges (D), and their corresponding quantification analysis (E). Values represent the mean $\pm$ SEM ($n = 6-8$ animals per group). *** $p < 0.005$ compared with the control group. O.D.: Optical Density.

Figure 8. Effect of acute alcohol intoxication (mean $\pm$ SEM blood alcohol level = 239 ± 0.13 mg/dL) on histone deacetylase (*HDAC*) gene expression in human peripheral blood. Data represent the mean $\pm$ SEM ($n = 20-22$ patients per group)

1 and the relative fold change obtained using the $2\Delta Ct$ method. * $p<0.05$,
2 *** $p<0.005$ compared with the control group.

3
4 **Figure 9.** Effects of operant alcohol self-administration on histone deacetylase
5 (*Hdac*) gene expression in rat peripheral blood at 60 min **before** (upper panel)
6 and 60 min **after** (lower panel) operant alcohol self-administration. Data
7 represent the mean $\pm$ SEM (n=10 animals per group) and the relative fold
8 change obtained using the $2\Delta Ct$ method. * $p<0.05$, ** $p<0.01$ compared with the
9 control group. Please note that a saccharine group was used as control.

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4 **Table 1.** Characteristics of patients (n = 20) with acute alcohol intoxication

Age (years)	20.4 (0.92)
Female sex	10 (50.0%)
Blood ethanol content (g/L)	2.39 (0.12)
Aspartate aminotransferase levels (IU/L)	23.44 (2.52)
Alanine aminotransferase levels (IU/L)	15.72 (0.99)
Alkaline phosphatase levels (IU/L)	73.67 (7.92)
γ - glutamyl transpeptidase levels (IU/L)	17.33 (1.41)
White blood cell count/ μ L	8550.53 (497.85)
Reported weekend drinking (n = 17)*	14 (82.4%)

5

6 Quantitative variables are presented as the mean (SEM) and qualitative variables are
7 presented as absolute frequencies (percentage). IU: international units. *Three patients
8 refused to answer the questionnaire regarding drinking patterns.

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Figure 1. Schematic representation of the experimental design. Red dots represent blood collected following cuts made to the tail veins of rats. White and blue rectangles represent cohorts of animals that were administered either water or alcohol and sham cut. Animal sacrifice was performed at two hours post alcohol binge to increase similarity in alcohol blood concentrations measured between rat and human subjects.
43x14mm (300 x 300 DPI)

Figure 2. Effects of one, four or eight alcohol binges on Class I histone deacetylase (Hdac) gene expression in rat peripheral blood, liver, heart, prefrontal cortex and amygdala. Data represent the mean $\pm$ SEM (n=10-12 animals per group) and the relative fold change obtained using the $2\Delta\Delta C_t$ method (see Methods section). *p<0.05 compared with the control group (no alcohol binge). 193x181mm (300 x 300 DPI)

Figure 3. Effects of one, four or eight alcohol binges on Class IIA histone deacetylase (Hdac) gene expression in rat peripheral blood, liver, heart, prefrontal cortex and amygdala. Data represent the mean $\pm$ SEM (n=10-12 animals per group) and the relative fold change obtained using the $2\Delta Ct$ method (see Methods section). *p<0.05 compared with the control group (no alcohol binge).
193x181mm (300 x 300 DPI)

Figure 4. Effects of one, four or eight alcohol binges on Class IIB and IV histone deacetylase (Hdac) gene expression in rat peripheral blood, liver, heart, prefrontal cortex and amygdala. Data represent the mean $\pm$ SEM (n=10-12 animals per group) and the relative fold change obtained using the $\Delta\Delta C_t$ method (see Methods section). *p<0.05 compared with the control group (no alcohol binge).

150x118mm (300 x 300 DPI)

Figure 5. Correlations among histone deacetylase (Hdac) gene expression in rat peripheral blood, liver, heart, prefrontal cortex (PFC) and amygdala (AMG) following variable alcohol binge conditions. Only correlations with significance lower than $p < 0.05$ are shown (green represents positive correlations, red represents negative; $n = 10-12$ animals per group).

117x108mm (300 x 300 DPI)

Figure 6. Blood alcohol concentrations over a four hour period following a weekly alcohol binge (3 g/kg) in rats. In the eighth week, blood samples were only collected at the 120 min time point following binge alcohol treatment, as animals were sacrificed at this time point. Values represent the mean of blood alcohol concentrations $\pm$ SEM (n=6 animals per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the first hour. # $p < 0.001$ compared with the first week (first alcohol binge).

98x75mm (300 x 300 DPI)

Figure 7. Upper panels depict 20x representative microphotographs of Oil Red O-Hematoxylin stained liver sections obtained from the control group of rats (A), and the group exposed to 8 alcohol binges (B). Lower panels depict Oil red O staining of lipid droplets of the control group (C), the group exposed to 8 alcohol binges (D), and their corresponding quantification analysis (E). Values represent the mean $\pm$ SEM (n=6-8 animals per group). ***p<0.005 compared with the control group. O.D.: Optical Density.
124x75mm (300 x 300 DPI)

Figure 8. Effect of acute alcohol intoxication (mean $\pm$ SEM blood alcohol level = 239 ± 0.13 mg/dL) on histone deacetylase (HDAC) gene expression in human peripheral blood. Data represent the mean $\pm$ SEM (n=20-22 patients per group) and the relative fold change obtained using the $2\Delta\Delta C_t$ method. * $p < 0.05$, *** $p < 0.005$ compared with the control group.
67x21mm (300 x 300 DPI)

Figure 9. Effects of operant alcohol self-administration on histone deacetylase (Hdac) gene expression in rat peripheral blood at 60 min before (upper panel) and 60 min after (lower panel) operant alcohol self-administration. Data represent the mean $\pm$ SEM (n=10 animals per group) and the relative fold change obtained using the $2\Delta\Delta C_t$ method. *p<0.05, **p<0.01 compared with the control group.

150x105mm (300 x 300 DPI)

Table S1. Details of the rat primers used for quantitative real-time PCR of each gene

	Gene	Name	GenBank accession no.	Direction	Primer Sequence (5'-3')	Amplicon Length (nt)
Internal control	18S	Rat 18S ribosomal RNA gene	M111188	Left	GGAGCCTGAGAAACGGCTA	64
				Right	TCGGGAGTGGGTAATTTGC	
Class I	Hdac1	Histone deacetylase 1, mRNA	NM_001025409	Left	GATCGGCTAGGTTGCTTCAA	131
				Right	CAGCACCAGCGCAGATTAC	
	Hdac2	Histone deacetylase 2, mRNA	NM_053447	Left	GCTGTCCTCGAGCTACTGAAA	110
				Right	GTCAACACGCGATCTGTTGT	
	Hdac3	Histone deacetylase 3, mRNA	NM_053448	Left	ATGCTGAAGAGAGGGGTCCT	64
				Right	TCATAGAATTCATTGGGTGCTTC	
Class II A	Hdac8	Histone deacetylase 8, mRNA	NM_001126373	Left	TGCCCTGCATAAACAATGA	83
				Right	GATAGGCATCAGTGTGGAAGG	
	Hdac4	Histone deacetylase 4, mRNA	NM_053449	Left	ATCACCATCCTTGCCCAAC	96
				Right	GGGAGAGCAAGCCTCTCAG	
	Hdac5	Histone deacetylase 5, mRNA	NM_053450	Left	TGCTGCCACTCAAGAGCA	103
				Right	ACAATGATGAAGCCAGAGG	
Class II B	Hdac7	Histone deacetylase 7, mRNA	XM_006226246	Left	AGCCCCAGTATCCCTACAG	117
				Right	ATGTTCCGGGGGTTTCAGT	
	Hdac9	Histone deacetylase 9, mRNA	NM_001200045	Left	TGCCCTTCTAGGAAATGAGC	76
				Right	AGCGGCTGCATTCTGATT	
Class II C	Hdac6	Histone deacetylase 6, mRNA	XM_003754740	Left	CAGACCACAGGAGCTTCACC	112
				Right	CGGATTCTTGGACTTCCTGA	
Class IV	Hdac10	Histone deacetylase 10, mRNA	NM_001035000	Left	AGCGATGTGTAGCCCATAGAG	75
				Right	ACCAGATGCTGCCTCCTTG	
Class V	Hdac11	Histone deacetylase 11, mRNA	NM_001106610	Left	ACGTCCTGGAGGGAGACC	69
				Right	CTTCATCCCTCTCACAATGC	

Supplemental Table 1 for rat primers
240x152mm (96 x 96 DPI)

Table S2. Details of the human primers used for quantitative real-time PCR of each gene						
	Gene	Name	GenBank accession no.	Direction	Primer Sequence (5'-3')	Amplicon Length (nt)
Internal control	ACTB	Human actin, beta, mRNA	NM_0011101	Left	CCAACCGCGAGAAGATGA	97
				Right	CCAGAGGCGTACAGGGATAG	
Class I	HDAC1	Human histone deacetylase 1, mRNA	NM_004964	Left	TAAATTCTTGCGCTCCATCC	102
				Right	AACAGGCCATCGAATACTGG	
	HDAC2	Human histone deacetylase 2, mRNA	NM_001527	Left	CAGTTGCTG GAGCTGTGAAG	139
				Right	AATTCAAGGATGGCAAGCAC	
	HDAC3	Human histone deacetylase 3, mRNA	NM_003883	Left	CTGGTCCTGCATTACGGTCT	130
				Right	TATTGGTGGGGCTGACTCTC	
	HDAC8	Human histone deacetylase 8, mRNA	NM_018486	Left	CGACGGAATTGAGCGTAT	138
				Right	TGTTCTGGGAAAAATCCTG	
Class II A	HDAC4	Human histone deacetylase 4, mRNA	NM_006037	Left	AGCAGAGGCTCTCCCTTTC	126
				Right	GTGGCTGCTCCAGTAAGACC	
	HDAC5	Human histone deacetylase 5, mRNA	XM_005256904	Left	AGATGCACTCTCCAGTGCT	102
				Right	GGATGATGGCAAATCCATT	
	HDAC7	Human histone deacetylase 7, mRNA	NM_001098416	Left	AGCGGAGGTGATTCTGAAAA	146
				Right	GAACAGGAGGCAAAAAGCTG	
	HDAC9	Human histone deacetylase 9, mRNA	NM_058176	Left	GCGGTCCAGGTTAAAACAGA	146
				Right	GACCAGAGCCTGGAGAATCG	
Class II B	HDAC6	Human histone deacetylase 6, mRNA	NM_006044	Left	GAACCTTGAGGCTGAAGCAC	138
				Right	TCCTGGATCAGTTGCTCCTT	
	HDAC10	Human histone deacetylase 10, mRNA	NM_032019.5	Left	TGGGAAGCTCCTGTACTCTT	73
				Right	GGCTGGAGTGGCTGTATAC	
Class IV	HDAC11	Human histone deacetylase 11, mRNA	NM_024827	Left	TTCTGTTTGAAGCGTGTGGAG	106
				Right	TACACACGCTTGTCGTCAT	

Supplemental Table 2 for human primers
246x152mm (96 x 96 DPI)