Spironolactone as a Potential New Pharmacotherapy for Alcohol Use Disorder: Convergent Evidence from Rodent and Human Studies

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Abstract

Evidence suggests that **spironolactone, a nonselective** mineralocorticoid receptor (MR) **antagonist**, modulates alcohol seeking and consumption. Therefore, spironolactone may represent a novel pharmacotherapy for alcohol use disorder (AUD). In this study**, we tested** the effects of spironolactone in a **mouse** model of alcohol drinking (drinking-in-the-dark) **and in a rat model of** alcohol dependence (vapor exposure). **We also investigated** the association between spironolactone receipt **for at least 60 continuous days** and change in self-reported alcohol consumption, using the Alcohol Use Disorders Identification Test-Consumption (AUDIT-C), in a pharmacoepidemiologic cohort study in the largest integrated healthcare system in the US. Spironolactone dose-dependently reduced the intake of sweetened or unsweetened alcohol solutions in male and female mice. No effects **of spironolactone** were observed on drinking of a non-alcohol-containing sweet solution, food or water intake, motor coordination, alcohol-induced ataxia, or blood alcohol levels. Spironolactone dose-dependently reduced operant alcohol self-administration in dependent and nondependent male **and female** rats. In humans, a greater reduction in alcohol consumption was observed among those who received spironolactone, compared to propensity-score matched individuals who did not receive spironolactone. The largest effects were among those who reported hazardous/heavy episodic alcohol consumption at baseline (AUDIT-C $\geq$ 8) and those exposed to $\geq$ 50 mg/day of spironolactone. These convergent findings across rodent and human studies **demonstrate** that spironolactone reduces alcohol use and support the hypothesis that this medication may be further studied as a novel pharmacotherapy for AUD.

Keywords: Alcohol, **aldosterone**, **steroid hormone**, **diuretic**, psychopharmacology, pharmacoepidemiology

1. Introduction

Alcohol use disorder (AUD) is a chronic relapsing brain disorder leading to high mortality, morbidity, and economic burden [1]. Compared to other chronic illnesses, currently available medications for AUD are limited. Therefore, there is a critical need to increase the armamentarium of pharmacotherapies to treat individuals with AUD [2]. Neuroendocrine systems involved in alcohol craving and drinking offer promising pharmacologic targets in this regard [3, 4].

The steroid hormone aldosterone and its related mineralocorticoid receptor (MR) regulate fluid and electrolyte homeostasis. **In response to decreased blood volume and/or blood pressure, aldosterone is secreted from the cortex of the adrenal gland and binds to MRs located in the principal cells of the kidney. This action facilitates sodium and water reabsorption into the blood and increases blood pressure.** MRs are also expressed in brain regions involved in AUD, including the amygdala, prefrontal cortex, and hippocampus [5-8], and modulate processes such as memory formation, fear extinction/recall, and stress responses [9-16]. Preliminary clinical and preclinical studies suggest that aldosterone and the MR play a role in alcohol seeking and consumption [17]. We previously reported that blood aldosterone concentrations are significantly decreased in actively drinking individuals with AUD who maintained alcohol abstinence during a 12-week outpatient follow-up [18]. Aldosterone levels positively correlated with self-reported alcohol craving and anxiety [18]. More recently, we provided evidence supporting the role of this endocrine system in alcohol use across three species [19]. In an alcohol self-administration model in monkeys, blood aldosterone levels increased from baseline to 6 and 12 months, and the MR gene (*Nr3c2*) expression levels in the central nucleus of the amygdala (CeA) were negatively correlated with average alcohol intake. The study also found a negative correlation between *Nr3c2* expression in the CeA and measures of anxiety-like behavior and compulsive-like drinking in alcohol-dependent rats. In a second, 12-week clinical study, participants who remained alcohol abstinent, compared to those not abstinent, had lower blood aldosterone concentrations at the endpoint, and aldosterone levels positively correlated with the amount of drinking, alcohol craving, and anxiety in the non-abstinent group [19]. Collectively, these

data suggest that higher MR signaling contributes to increased alcohol consumption, and medications that block MR may represent a novel pharmacotherapeutic approach for AUD.

Spironolactone is an FDA-approved MR antagonist medication used to treat essential hypertension, heart failure, edema, primary hyperaldosteronism, and hypokalemia. Spironolactone has been tested in preclinical studies of alcohol drinking and seeking with inconsistent results. Systemic or intracerebroventricular administration of spironolactone, or the MR antagonist RU28318, did not reduce alcohol drinking in male rats [20, 21] or mice of both sexes [22] tested on a continuous (24 h) two-bottle (water *vs.* alcohol) choice model nor on a limited (1 h) two-bottle choice model following fluid restriction [23]. Limitations of these studies included low blood alcohol concentration achieved with continuous two-bottle choice models and a "physiological fluid need" (i.e., water restriction) [24]. However, Kashkin and colleagues reported that seven days of oral spironolactone treatment decreased alcohol drinking (and blood pressure) in high drinking, but not low drinking, male rats given continuous two-bottle choice access [25]. More recently, following the renewed interest in the aldosterone/MR pathway in AUD [19], Makhijani and colleagues reported that systemic injection of spironolactone reduced operant alcohol self-administration in both male and female rats, and suppressed the persistence of alcohol responding under an extinction condition in female rats [26]. However, spironolactone decreased general locomotion, especially in males, and this may have affected responding for alcohol. In a follow-up study, Makhijani and colleagues found that MR antagonism with eplerenone or MR knockdown in the CeA **transiently** reduced alcohol self-administration in female rats [27].

Given these promising but inconsistent results, additional studies are needed to better understand the pharmacologic potential of spironolactone in reducing alcohol consumption under different experimental settings, including binge-like and **alcohol dependence-associated drinking**. Additionally, studies are needed to determine to what extent spironolactone, *per se* or in combination with alcohol, impairs locomotion and motor coordination, and whether there is an interaction between spironolactone and alcohol pharmacokinetics. Finally, initial bench-to-bedside translation of the potential role of spironolactone in

AUD is critically needed. We recently conducted a pharmacoepidemiologic study, using electronic health record data from Kaiser Permanente Northern California. Over 500 individuals treated with spironolactone, for any indication, were propensity score-matched with untreated controls, and the change in weekly alcohol use from baseline to follow-up was compared. Results showed a greater reduction in alcohol drinking among individuals who received spironolactone than those who did not. A significant dose-response relationship was also found, providing clinically relevant data in support of spironolactone for the treatment of AUD [28]. Although this study was promising, independent replication is necessary to confirm these findings, ideally in larger sample sizes and using different methodologies.

The aim of the present study was to address these gaps in knowledge, by testing the effect of spironolactone on alcohol-related behaviors in mice and rats and by conducting a pharmacoepidemiologic study using data from the largest integrated healthcare system in the US. Our hypothesis was that spironolactone would decrease alcohol consumption in rodents, without affecting their general consummatory behavior, causing sedation/motor incoordination, or affecting alcohol-induced ataxia and blood alcohol levels. Further, we hypothesized that patients prescribed spironolactone would display a reduction in their self-reported alcohol consumption, compared to propensity score-matched individuals.

2. Methods and Materials

2.1. Spironolactone and alcohol use in rodents: Psychopharmacology studies

Full methodological details are described in **Appendix 1**. Briefly, adult male and female C57BL/6J mice were used to test the effects of spironolactone (0, 25, 50, 100, 200 mg/kg; injected 30 min before alcohol drinking) on binge-like alcohol consumption (drinking-in-the-dark, DID) [29]. We also assessed food and water intake, blood alcohol levels, motor coordination (rotarod test), and spontaneous locomotion (circular corridor test) in mice. Adult male **and female** Wistar rats were used to test the effects of spironolactone

injections (0, 25, 50, and 75 mg/kg; injected 60 min before alcohol drinking) on operant alcohol self-administration (fixed-ratio 1, FR1) in alcohol-dependent (intermittent alcohol vapor exposure) and nondependent (exposed to air without alcohol) rats [30]. We also assessed blood alcohol levels and motor coordination (rotarod test) in male rats. Doses of spironolactone tested in these rodent experiments were selected based on previous studies and body surface area of species [26, 27, 31].

2.2. Spironolactone and alcohol use in humans: A pharmacoepidemiology study

Full methodological details are described in **Appendix 2**. Briefly, an observational cohort study was conducted, using electronic health record data from the US Department of Veterans Affairs (VA), to examine the association between spironolactone receipt (at least 60 continuous days) and change in self-reported alcohol consumption (Alcohol Use Disorders Identification Test-Consumption, AUDIT-C) [32, 33]. Each spironolactone exposed patient was propensity score matched [34, 35] to up to five unexposed patients, using a greedy matching algorithm [36]. Multivariable difference-in-difference (Diff-in-Diff) linear regression models [37, 38] estimated the differential change between baseline (pre-index) and follow-up (post-index) AUDIT-C scores **among exposed and unexposed patients**. Subgroup analyses stratified by baseline AUDIT-C and average daily dose of spironolactone were also performed.

3. Results

3.1. Effects of spironolactone in rodents

3.1.1. Effect of spironolactone on alcohol and non-alcohol-containing solution drinking in mice

For mice drinking a sweetened alcohol solution (n = 15), two-way repeated measures ANOVA revealed a main effect of dose ($F_{4,52}$ = 9.09, p < 0.0001) and sex ($F_{1,13}$ = 6.05, p = 0.02; female > male), but there was

no interaction between sex and dose ($F_{4,52}$ = 0.42, p = 0.78). The Dunnett *post hoc* comparisons indicated that spironolactone at doses of 50 mg/kg (p = 0.007), 100 mg/kg (p = 0.002) and 200 mg/kg (p < 0.0001) significantly reduced alcohol intake (**Figure 1A**). In mice drinking an unsweetened alcohol solution, two-way repeated measures ANOVA revealed a main effect of dose ($F_{4,52}$ = 5.77, p = 0.0006), but no main effect of sex ($F_{1,13}$ = 1.41, p = 0.25) and no interaction between sex and dose ($F_{4,52}$ = 1.26, p = 0.29). Dunnett *post hoc* analyses revealed that spironolactone significantly reduced alcohol intake at doses of 50 mg/kg (p = 0.04), 100 mg/kg (p < 0.0001) and 200 mg/kg (p = 0.02; **Figure 1B**). ANOVA of drinking data in mice receiving a non-alcohol-containing sweet solution showed a main effect of dose ($F_{4,52}$ = 2.61, p = 0.04). However, the Dunnett *post hoc* test revealed that none of the tested spironolactone doses significantly reduce non-alcohol-containing sweet solution intake, compared to the vehicle condition. Furthermore, the ANOVA did not show a sex effect ($F_{1,13}$ = 0.66, p = 0.42) or an interaction between sex and dose ($F_{4,52}$ = 2.10, p = 0.09). A female mouse from the non-alcohol-containing sweet group was identified as a significant outlier, so the drinking data for this animal was excluded (**Figure 1C**).

- Figure 1 here -

3.1.2. Effect of spironolactone on food and water intake in mice

Chow and water intake of mice were evaluated at 6 h and 24 h post-treatment with vehicle or spironolactone (200 mg/kg). Three-way repeated measures ANOVA on chow intake revealed a main effect of time ($F_{1,11}$ = 74.98, p < 0.0001) and of sex ($F_{1,11}$ = 11.70, p = 0.0057), but no main effect of dose ($F_{1,11}$ = 0.01, p = 0.90) nor an interaction between time and dose ($F_{1,11}$ = 0.18, p = 0.67) or sex and dose ($F_{1,11}$ = 0.70, p = 0.41). A significant interaction between time and sex ($F_{1,11}$ = 6.99, p = 0.02) was detected. The Holm-Sidak *post hoc* test indicated that females consumed more chow than males 24 h post-treatment, regardless of whether they were treated with vehicle or 200 mg/kg of spironolactone (**Table 1A**).

Similar results were found for water intake. Three-way repeated measures ANOVA revealed a main effect of time ($F_{1,11}$ = 123.30, p < 0.0001) and of sex ($F_{1,11}$ = 6.10, p = 0.03), but no main effect of dose ($F_{1,11}$ = 1.24, p = 0.28) and no interaction between time and dose ($F_{1,11}$ = 0.89, p = 0.36) or sex and dose ($F_{1,11}$ = 1.15, p = 0.30). A significant interaction between time and sex ($F_{1,11}$ = 15.10, p < 0.002) was observed. *Post hoc* analyses indicated that female mice drank significantly more water than male mice at 24 h, regardless of treatment (**Table 1B**).

- Table 1 here -

3.1.3. Effect of spironolactone on motor coordination and blood alcohol levels in mice

Rotarod performance of spironolactone-treated (200 mg/kg) mice (n = 11) did not significantly differ from vehicle-treated mice at any timepoints. Three-way repeated measures ANOVA did not reveal a main effect of drug ($F_{1,9}$ = 0.90, p = 0.36), time ($F_{2,18}$ = 0.17, p = 0.84) or sex ($F_{1,9}$ = 2.20, p = 0.17) on rotarod performance for the saline condition, i.e., the mice received a saline injection 30 min before rotarod testing. No interaction was found between dose and time ($F_{2,18}$ = 0.80, p = 0.46), dose and sex ($F_{1,9}$ = 0.76, p = 0.40), time and sex ($F_{2,18}$ = 2.27, p = 0.13), nor between all three of these factors ($F_{2,18}$ = 0.14, p = 0.86), indicating that spironolactone *per se* did not disrupt motor coordination (**Figure 2A**).

A three-way repeated measures ANOVA revealed a main effect of time ($F_{5,45}$ = 95.97, p < 0.0001) on rotarod performance of spironolactone-treated (200 mg/kg) mice (n = 11) during conditions of alcohol-induced ataxia, i.e., the mice received an alcohol (1.5 g/kg) injection 30 min before rotarod testing. This observation indicates that alcohol caused motor incoordination in mice and that this effect ameliorates over time. However, the ANOVA did not reveal a main effect of drug ($F_{1,9}$ = 0.78, p = 0.40) nor an interaction between drug and time ($F_{5,45}$ = 1.56, p = 0.18), indicating that spironolactone did not affect the ataxic effects of alcohol (**Figure 2B**).

Immediately following the rotarod trials at 30 min and 90 min, blood was collected for blood alcohol levels measurements. Three-way repeated measures ANOVA revealed a main effect of time ($F_{1,9} = 223.90$, $p < 0.0001$), indicating that blood alcohol levels decreased over time. The ANOVA did not show a main effect of drug ($F_{1,9} = 0.64$, $p = 0.44$) nor an interaction between drug and time ($F_{1,9} = 0.23$, $p = 0.63$), indicating that spironolactone did not affect alcohol elimination (**Figure 2C**). No main effect of sex on alcohol-induced ataxia ($F_{1,9} = 0.31$, $p = 0.58$; **Figure 2B**) or blood alcohol levels ($F_{1,9} = 1.33$, $p = 0.27$; **Figure 2C**) was found.

3.1.4. Effect of spironolactone on spontaneous locomotion in mice

Grubb's test indicated that one female mouse was a significant outlier, and data from this mouse was excluded. Two-way repeated measures ANOVA showed no main effect of dose ($F_{2,24} = 0.80$, $p = 0.45$) or sex ($F_{1,12} = 0.13$, $p = 0.71$) on total distance traveled (m) on the circular corridor, and no dose × sex interaction ($F_{2,24} = 0.18$, $p = 0.83$; **Figure 2D**).

- Figure 2 here -

3.1.5. Effect of spironolactone on alcohol self-administration, motor coordination, and blood alcohol levels in dependent and nondependent rats

An unpaired Student's t-test showed a significant difference in the average number of lever presses for alcohol between alcohol-dependent ($n = 12$) and nondependent ($n = 12$) male rats over the last three self-administration sessions preceding spironolactone treatment ($t_{22} = 6.7$, $p = 0.0001$). A two-way repeated measures ANOVA showed a significant spironolactone effect (main effect of dose: $F_{3,66} = 43.95$, $p < 0.0001$). The Dunnett *post hoc* test indicated that spironolactone at 25 mg/kg ($p < 0.0001$), 50

mg/kg ($p < 0.0001$), and 75 mg/kg ($p < 0.0001$) reduced alcohol self-administration in both alcohol-dependent and nondependent male rats (**Figure 3A). The ANOVA did not show a significant effect of alcohol dependence ($F_{1,22} = 2.17$, $p = 0.53$) nor a significant interaction between spironolactone and alcohol dependence ($F_{3,66} = 0.74$, $p = 0.90$**).

A three-way repeated measures ANOVA revealed a main effect of time ($F_{4,88} = 40.99$, $p < 0.0001$), a main effect of group ($F_{1,22} = 11.41$, $p = 0.002$), and a time × group interaction ($F_{4,88} = 6.63$, $p < 0.0001$) on rotarod performance of dependent ($n = 9$) and nondependent ($n = 15$) male rats during conditions of alcohol-induced ataxia. These results indicate that alcohol caused motor incoordination in both groups of rats, but alcohol-dependent rats showed tolerance to the ataxic effect of alcohol, compared with nondependent rats. However, the ANOVA did not reveal a main effect of spironolactone treatment ($F_{1,22} = 0.38$, $p = 0.54$) nor an interaction between spironolactone and time ($F_{4,88} = 1.42$, $p = 0.23$), indicating that spironolactone had no effect on alcohol-induced ataxia in male rats **(Figure 3B).**

Regarding blood alcohol levels, a three-way repeated measures ANOVA revealed a main effect of time ($F_{3,66} = 17.97$, $p < 0.0001$), indicating that blood alcohol levels decreased over time. There was no main effect of group ($F_{1,22} = 0.15$, $p = 0.70$), spironolactone treatment ($F_{1,22} = 0.003$, $p = 0.95$) nor an interaction between spironolactone and time ($F_{3,66} = 0.67$, $p = 0.56$), indicating that alcohol-dependent and nondependent male rats achieved similar blood alcohol levels across timepoints, following a 1.5 g/kg alcohol injection, and that spironolactone did not affect the elimination of alcohol (**Figure 3C**).

An unpaired Student's t-test showed a significant difference in the average number of lever presses for alcohol between alcohol-dependent ($n = 8$) and nondependent ($n = 7$) female rats over the last three self-administration sessions preceding spironolactone treatment ($t_{13} = 5.7$, $p = 0.0001$). A two-way repeated-measures ANOVA showed a significant effect of alcohol dependence (main effect of group: $F_{1,13} = 15.19$, $p = 0.002$) and a significant spironolactone effect (main effect of dose: $F_{3,39} = 12.06$, $p < 0.0001$). The Dunnett's *post hoc* test indicated that spironolactone at 50 mg/kg ($p = 0.02$)

and 75 mg/kg ($p < 0.0001$) reduced alcohol self-administration in both dependent and nondependent female rats (Figure 3D). The ANOVA did not show a significant interaction between spironolactone and alcohol dependence ($F_{3,39} = 0.99$, $p = 0.41$).

- Figure 3 here -

3.2. Effects of spironolactone receipt in humans

3.2.1. Sample

We identified 30,939 spironolactone exposed and 2,083,402 unexposed individuals who reported any alcohol consumption in the two years prior to index date. A total 20,382 exposed patients were matched; however, 9,656 (47%) did not have a follow-up AUDIT-C and were unable to be included in analysis. Among those in the final matched cohort, 3,016 (28.1%) were matched to five unexposed individuals, 2,287 (21.3%) to four, 1,541 (14.4%) to three, 1,728 (16.1%) to two, and 2,154 (20.1%) to one unexposed individual. Thus, the matched cohort consisted of 10,726 exposed and 34,461 unexposed individuals.

Before propensity score matching, the distribution of baseline characteristics differed between exposed and unexposed individuals (**Table 2**). Consistent with current indications for spironolactone, those who received spironolactone had a higher prevalence of hepatic decompensation (14.6% *vs.* 0.3%), coronary artery disease (38.0% *vs.* 13.5%), diabetes (39.6% *vs.* 21.3%), chest pain (38.8% *vs.* 22.3%), and chronic medication use (39.6% *vs.* 7.5% with ≥11 medications), compared to the unexposed group. After propensity score matching, differences were minimized between the two treatment groups (all standardized mean differences ≤0.2, with most ≤0.1) [39]. Thus, matching produced treatment groups that were considered well balanced (**Table 2**). Among exposed individuals in the matched cohort, 25%, 57%, and 18% were prescribed daily doses of spironolactone <25 mg/day, 25-49 mg/day, and ≥50 mg/day, respectively. Median follow-up time was 542 days (IQR 337-730 days).

- Table 2 here -

3.2.2. Changes in alcohol consumption

Overall, AUDIT-C scores decreased during the study period in both treatment groups. Average AUDIT-C scores decreased from 3.07 (standard deviation [SD] 0.02) to 2.16 (SD 0.02) among exposed individuals and from 2.96 (SD 0.01) to 2.22 (SD 0.01) among unexposed individuals (**Table 3**). Therefore, on average, AUDIT-C scores decreased 0.17 points more among spironolactone exposed individuals, compared to the unexposed individuals (Diff-in-Diff -0.17 points, 95% CI -0.09, -0.25; $p < 0.0001$). In the analysis stratified by baseline AUDIT-C, average scores decreased 0.07 points (95% CI -0.01, -0.14; $p = 0.02$), 0.13 points (95% CI -0.02, -0.24; $p = 0.02$), and 0.47 points (95% CI -0.29, -0.66; $p < 0.0001$) more among exposed, compared to unexposed individuals, in those with baseline AUDIT-C scores of 1-3, 4-7, and ≥8, respectively, indicating that the largest effect was observed in the group with highest severity of alcohol use at baseline. Similarly, the analysis stratified by average spironolactone dosage found the largest Diff-in-Diff estimate among individuals exposed to ≥50 mg/day of spironolactone (Diff-in-Diff: -0.69 points, 95% CI: -0.50, -0.89; $p < 0.0001$) **(Figure 4)**.

- Table 3 and Figure 4 here -

4. Discussion

The present findings provide translational evidence across three species (mice, rats, and humans) supporting the hypothesis that spironolactone represents a promising pharmacological treatment for AUD. Using a mouse model of alcohol drinking [29], we observed that spironolactone dose-dependently decreased the consumption of sweetened and unsweetened alcohol solutions in male and female mice. Although female mice drank significantly more sweetened alcohol than male mice, spironolactone was equally effective in

both sexes. Of note, spironolactone had no effect on consumption of a non-alcohol-containing sweet solution and on food or water intake. Spironolactone *per se* did not affect spontaneous locomotion (circular corridor) or motor coordination (rotarod) and did not interfere with alcohol-induced ataxia (rotarod) or blood alcohol levels in mice. These findings suggest that spironolactone did not change motor and consummatory behaviors in general or alcohol pharmacokinetics. Using a rat model of alcohol dependence [30], we observed that spironolactone decreased operant alcohol self-administration (fixed-ratio 1 schedule of reinforcement) **in dependent and nondependent male and female rats**. Spironolactone did not affect motor coordination in alcohol-dependent and nondependent male rats and did not reverse the already established tolerance to the alcohol-induced ataxia in dependent **male** rats. More specifically, compared with nondependent rats, alcohol vapor-exposed dependent rats exhibited less motor impairment under alcohol intoxication compared with nondependent rats, and this was not affected by spironolactone treatment. Consistent with these preclinical results, our pharmacoepidemiologic study indicated that individuals who received spironolactone for any indication reported greater reduction in alcohol drinking than matched controls who did not receive spironolactone.

We used the DID test as a model of alcohol binge-like drinking in mice and used both unsweetened alcohol and sweetened alcohol solutions, which lead to different levels of alcohol drinking. When mice were given the sweetened alcohol solution, they consumed on average approximately 3-4 g/kg of alcohol in 4 h, which is reported to produce blood alcohol levels approaching 0.08 g/dL [31]. This drinking level is considered by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as alcohol 'binge drinking', which increases risky behaviors, the development of chronic illnesses, and the risk of developing AUD [40]. The use of a sweetened alcohol solution also resembles the types of alcohol containing beverages commonly consumed by humans. To test the specificity of the spironolactone effect to alcohol, we tested the effects of spironolactone on the consumption of an unsweetened alcohol solution and a sweetened solution without alcohol.

Our results of decreased alcohol drinking in mice given spironolactone are consistent with previous studies in rats that operantly self-administrated a sweetened alcohol (15-20% alcohol + 2% sucrose) solution [26, 27] or rats that were given two-bottle choice between unsweetened alcohol (9%) and water [25]. However, spironolactone did not affect drinking of a non-alcohol-containing sweetened solution or spontaneous locomotion in mice of both sexes herein. In contrast, spironolactone decreased self-administration of a non-alcohol-containing sucrose solution in male rats and locomotion in male and female rats in previous work [26]. We also did not observe an effect of spironolactone on motor performance on the rotarod, indicating that spironolactone *per se* did not cause ataxia. A previous study reported that MR inactivation in the forebrain of male and female mice had no impact on rotarod performance [41]. Also important for our data interpretation is that spironolactone did not influence alcohol-induced ataxia on the rotarod nor blood alcohol levels in mice of both sexes. A previous study showed that repeated spironolactone treatment did not affect alcohol (1.5 g/kg)-induced motor impairment (hanging on a rod with front paws) in male rats or blood alcohol levels [42]. We also provide novel evidence that spironolactone decreased alcohol intake in male **and female** rats that were made dependent on alcohol via chronic, intermittent alcohol vapor exposure [43] and allowed to operantly self-administer unsweetened alcohol. **In this model of alcohol dependence, the rats exhibit motivational and somatic signs of withdrawal [59], analogous to those observed in humans with AUD.** However, spironolactone did not alter already established alcohol tolerance **in male rats suggesting that the effect of spironolactone on alcohol seeking is not via a reversal of tolerance in general [60].** Together, the present results and prior studies provide complimentary evidence that spironolactone reduces nondependent drinking, binge-like drinking, and dependent drinking, without affecting alcohol tolerance or motor and consummatory behaviors in general.

In parallel, we conducted a pharmacoepidemiologic cohort study to translate our rodent findings to humans and observed consistent results, i.e., a significant association between spironolactone treatment and reduction in self-reported alcohol consumption. As is typical in a middle-aged cohort of patients, AUDIT-C scores decreased over time in both spironolactone-exposed and unexposed individuals; however, there

was a significantly greater decrease in the scores of individuals exposed to spironolactone. Consistent with our rodent studies, we found a dose-dependent effect of spironolactone in humans, which suggests a potential causal relationship between spironolactone dose and change in alcohol drinking. Specifically, individuals exposed to ≥50 mg/day of spironolactone had a significantly greater decrease in AUDIT-C scores than those exposed to <50 mg/day. **Biological gradient, i.e., dose-response relationship, is one of the Hill's criteria for causation in traditional epidemiology [44]. When an incremental change of the exposure leads to a respective incremental change of the outcome, like the relationship observed in this study between spironolactone dose and change in AUDIT-C score, a causal relationship may be assumed, although other potentially confounding factors should be considered [45].** These findings are consistent with, and substantially extend, a recent retrospective cohort study comparing 523 spironolactone-treated adults and 2,305 untreated adults using electronic health record data from Kaiser Permanente Northern California (KPNC) [28]. First, the present data are derived from a much larger patient population, and different in terms of geography (national *vs.* regional) and demographics (younger and predominantly male), compared to the KPNC cohort. Of note, US Veterans have an increased likelihood of developing AUD [46]; therefore, the present human results are particularly relevant as they were generated in **a cohort at higher risk of AUD**. Second, we analyzed a different alcohol-related outcome (AUDIT-C), which is important and clinically relevant given that AUDIT-C scores have been associated with alcohol-related medical consequences, including alcohol dependence [47] and mortality [48]. **AUDIT-C scores have been routinely collected in the VA since 2008, providing the longitudinal data necessary for our analysis.** Third, herein we analyzed a sample size that was ~20 times larger for the spironolactone-treated group and ~15 times larger for the untreated group. **Finally, the present study had a longer follow-up period (~2 years), compared to our previous study (~6 months).** Thus, the present human data, together with those in Palzes et al. [28], provide strong pharmacoepidemiology-based evidence supporting a role of spironolactone in AUD.

The mechanism(s) of action by which spironolactone reduces alcohol consumption is an area of current investigation. We hypothesize that increased levels of circulating aldosterone may contribute to alcohol drinking by increasing anxiety, facilitating brain stress system activation, and/or inducing neuroinflammation. Alterations in the levels of hormones that regulate fluid and electrolyte homeostasis, including aldosterone, have been proposed to accompany and potentially contribute to AUD [3]. Aldosterone levels significantly correlated with alcohol withdrawal [49], anxiety, obsessive craving [18, 19], and alcohol drinking [19] in patients with AUD. Primary aldosteronism in humans was associated with increased anxiety [50, 51], and chronic treatment with aldosterone increased anxiety-like behavior in rats [52]. **Many drugs that have shown promise in treating AUD (e.g., baclofen, gabapentin, pregabalin) also have anxiolytic effects [2, 53], and it is conceivable that at least part of spironolactone's effect on alcohol use may be driven by anxiety reduction [54-57].** Moreover, in adrenalectomized rats, aldosterone increased corticotropin-releasing factor (CRF) mRNA levels in the paraventricular nucleus of the hypothalamus and in the CeA [58]. Increased CRF activity in limbic areas, particularly the amygdala, drives **negative emotional states** and drinking associated with alcohol dependence in rodent models [59-62].

The exact role of MR in stress and alcohol drinking is intriguingly multifaceted. In the brain, MR expression is enriched in limbic regions, such as the prefrontal cortex, hippocampus, and extended amygdala, as well as the nucleus of the solitary tract [63]. Brain MRs are involved in various cognitive processes, regulate basal hypothalamic-pituitary-adrenal (HPA) axis activity, and mediate the autonomic and HPA axis response to stress, which are dysregulated in alcohol dependence [62]. Although cortisol/corticosterone (CORT) binds MR with high affinity and its concentration exceeds that of aldosterone, aldosterone activity in the brain in the presence of corticosteroid has been reported [64]. Because MRs in the brain are almost completely occupied at basal circadian CORT levels, it is hypothesized that receptor turnover plays an important role in MR function, as well as the existence of a membrane-localized MR, and the formation of steroid receptor heterodimers, such as MR-glucocorticoid receptor (GR) heterodimers, during gene transcription that may all facilitate different outcomes of MR activation in various physiological states.

Thus, increased blood aldosterone may be expected to increase brain MR signaling, and perhaps GR signaling, the latter of which has been shown to be involved in alcohol dependence in rodents and humans [65] and blocked by spironolactone.

There is also evidence that enhanced brain MR activity in brain regions mediating emotional responses is beneficial for a healthy state and may be protective in the face of stress and possibly psychiatric illness. High expression of MRs is associated with enhanced cognitive function, usage of active coping strategies, and resistance to chronic stress-induced cognitive dysfunction [66]. **Consistent with the role of MR activity in cognition [9], both preclinical and clinical studies indicate that MR blockade may negatively impact certain cognitive domains, such as selective attention, visuospatial memory, reversal learning, and decision making [41, 67-72]. Such effects are more likely with repeated and continuous MR blockade, as opposed to acute spironolactone doses that we used in our rodent experiments. Future preclinical and human studies should incorporate cognitive assessments over time to examine the extent to which spironolactone, or other MR antagonists, combined with alcohol, may produce cognitive deficits.** Decreased MR expression was observed in post-mortem brains of individuals with depression [73], and MR expression was increased by antidepressant administration in rats [74]. MR agonism with fludrocortisone enhanced cognitive function and antidepressant efficacy in humans [75, 76]. Similarly, we identified a relationship between decreased MR expression and alcohol dependence. Decreased MR in the CeA was correlated with compulsive-like alcohol drinking in dependent rats and increased alcohol drinking in monkeys [19].

Altogether, the present study and previous findings indicate dysregulations in the aldosterone/MR pathway in alcohol dependence, but whether the effects of spironolactone are peripheral, central, or both remain to be demonstrated. Acute MR antagonism with eplerenone or downregulation of MR in the amygdala decreased alcohol drinking in rats [27], supporting a central effect. Spontaneously hypertensive rats, which are typically high alcohol drinkers [77], exhibited increased MR binding capacity and expression in the brain and peripheral organs (for review, see: [78]). In normotensive high drinking rats, chronic treatment

with spironolactone decreased both alcohol drinking and blood pressure [25], potentially suggesting a parallel central and peripheral effect.

The extent to which spironolactone effects are specifically MR-mediated **remains unclear**. Spironolactone and its metabolites are non-selective MR antagonists, such that they also bind GRs, progesterone, and androgen receptors [79, 80]. Chronic treatment with aldosterone increased proinflammatory cytokine expression in the mouse brain and this effect was blocked by spironolactone [81]. Another target of spironolactone is pannexin 1 channels, which regulate adenosine triphosphate and, thereby, contribute to many physiological processes, particularly cardiovascular function [82]. Spironolactone via pannexin 1 channel inhibition rapidly lowers blood pressure and inhibits α_1-adrenergic activity in mice [83]. We have recently reported that the pannexin 1 channel inhibitor probenecid reduced alcohol drinking in nondependent and dependent rats, as well as binge-like drinking in mice [84]. **Overall, future studies are needed to elucidate the exact mechanism(s) of action of spironolactone on alcohol-related outcomes.**

Spironolactone is a widely used medication for a variety of indications, mostly related to cardiovascular diseases and hemodynamic disturbances in patients with chronic disorders (e.g., patients with liver cirrhosis or nephrotic syndrome). As such, a strength of this work is that we are testing a medication with known tolerability, safety, and side effect profiles, even in individuals with severe chronic diseases. AUD leads to several chronic diseases, such as alcohol-related liver and cardiovascular diseases [85, 86], and there is certainly a need to identify effective and safe medications to treat patients with AUD and comorbid disease [87, 88]. Future prospective human studies are needed not only to test the putative efficacy of spironolactone in AUD via double-blind, placebo-controlled, randomized, clinical trials, but also to confirm its safety and tolerability in individuals with AUD. Potential drug-alcohol interactions will also need to be further addressed in humans. **Based on the observation that spironolactone reduced alcohol self-administration in alcohol-dependent and nondependent rats, in mice exhibiting a binge-like level of alcohol drinking, and in mice that consumed a relatively lower amount of alcohol, we suggest that spironolactone may play a general role in alcohol reinforcement, although our**

pharmacoepidemiologic data suggested a higher effect of spironolactone in those with higher baseline drinking. Additional preclinical and clinical studies on alcohol consumption as well as other aspects associated with AUD such as craving/relapse, and reward and stress function [89-91] will help with the understanding of spironolactone's effects observed in the present study. Furthermore, due to the heterogeneity of AUD**, which likely influences the** effect sizes of medications in clinical studies [2], it will be important for future work to identify potential biomarkers of **spironolactone efficacy in** sub-populations with AUD.

In conclusion, the present study provides converging evidence, across psychopharmacologic experiments in mice and rats and pharmacoepidemiologic observations in humans, supporting that spironolactone represents a promising pharmacological treatment for AUD. These findings collectively support future perspective randomized, controlled studies testing spironolactone in patients with AUD, as well as additional work to understand the mechanisms related to the role of the MR in AUD and how spironolactone reduces alcohol drinking.

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Table 1. Spironolactone did not affect (**A**) chow or (**B**) water intake in mice.

		Vehicle-Treated				Spironolactone-Treated			
		Males		Females		Males		Females	
A	**Total Chow** Intake (g)**	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*
	6 h	0.95	*0.172*	1.28	*0.235*	0.43	*0.197*	1.22	*0.397*
	24 h$^{\#\#\#\#}$	2.51	*0.343*	4.00†	*0.259*	2.11	*0.404*	4.00†	*0.886*
B	**Total Water* Intake (mL)**	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*
	6 h	0.80	*0.096*	1.06	*0.225*	0.80	*0.177*	0.98	*0.328*
	24 h$^{\#\#\#\#}$	1.69	*0.164*	3.16††	*0.293*	1.68	*0.263*	2.54	*0.572*

Male: $n = 8$; Female: $n = 5$. *$p < 0.05$, **$p < 0.01$, male *vs.* female (overall sex effect; three-way ANOVA). $^{\#\#\#\#}p < 0.0001$, 6 h *vs.* 24 h (overall time effect; three-way ANOVA). $^{\dagger}p < 0.05$, male vehicle-treated 24 h *vs.* female vehicle-treated 24 h and male spironolactone-treated 24 h *vs.* female-spironolactone treated 24 h, $^{\dagger\dagger}p < 0.01$, male vehicle-treated 24 h *vs.* female vehicle-treated 24 h (sex *vs.* time interaction; three-way repeated measures ANOVA followed by Holm-Sidak *post hoc* test).

Table 2. Distribution of baseline characteristics in spironolactone exposed and unexposed individuals before and after propensity score (PS) matching.

	Full cohort			Matched cohort		
	Exposed	Unexposed		Exposed	Unexposed	
Characteristic	**n = 30,939**	**n = 2,083,402**	**SMD**	**n = 10,726**	**n = 10,726***	**SMD**
Age (years)						
<55	5,389 (17.4)	549,459 (26.4)	0.19	1,683 (15.7)	1,716 (16.0)	0.03
55-59	6,747 (21.8)	425,267 (20.4)		2,059 (19.2)	2,171 (20.2)	
60-64	12,016 (38.8)	682,783 (32.8)		4,227 (39.4)	4,198 (39.1)	
≥65	6,787 (21.9)	425,893 (20.4)		2,757 (25.7)	2,641 (24.6)	
Race/ethnicity						
White	20,573 (66.5)	1,413,221 (67.8)	0.13	7,317 (68.2)	7,195 (67.1)	0.06
Black	7,136 (23.1)	391,367 (18.8)		2,401 (22.4)	2,459 (22.9)	
Hispanic	1,315 (4.3)	108,479 (5.2)		357 (3.3)	480 (4.5)	
Other	704 (2.3)	61,807 (3.0)		253 (2.4)	231 (2.2)	
Missing	1,211 (3.9)	108,528 (5.2)		398 (3.7)	361 (3.4)	
Male sex	29,540 (95.5)	1,958,068 (94.0)	0.06	10,229 (95.4)	10,177 (94.9)	0.03
HCV+	4,822 (15.6)	119,706 (5.8)	0.33	979 (9.1)	1,290 (12.0)	0.06
AUD						
Never	20,398 (65.9)	1,676,893 (80.5)	0.32	7,503 (70.0)	7,417 (69.2)	0.07
Lifetime	3,162 (10.2)	187,489 (9.0)		1,202 (11.2)	1,389 (13.0)	
Current	7,379 (23.9)	219,020 (10.5)		2,021 (18.8)	1,920 (17.9)	
Substance use treatment program visit	9,108 (29.4)	323,974 (15.6)	0.31	2,095 (19.5)	1,912 (17.8)	0.05
Any hospitalization	9,976 (32.2)	169,306 (8.1)	0.64	2,873 (26.8)	2,280 (21.3)	0.18
Coronary artery disease	11,743 (38.0)	281,078 (13.5)	0.55	4,673 (43.6)	3,983 (37.1)	0.16
Diabetes	12,255 (39.6)	444,048 (21.3)	0.38	4,793 (44.7)	4,631 (43.2)	0.04
Hepatic decompensation	4,515 (14.6)	5,492 (0.3)	0.57	201 (1.9)	209 (1.9)	0.06
Hyperlipidemia	19,366 (62.6)	1,139,384 (54.7)	0.01	7,676 (71.6)	7,181 (67.0)	0.08
Abdominal pain	11,201 (36.2)	512,747 (24.6)	0.19	3,693 (34.4)	3,588 (33.5)	0.04
Chest pain	12,017 (38.8)	464,716 (22.3)	0.30	4,498 (41.9)	4,015 (37.4)	0.11
Any chronic pain	26,863 (86.8)	1,722,836 (82.7)	0.01	9,466 (88.3)	9,590 (89.4)	0.03
Number of medications						
≤5	6,163 (19.9)	1,553,268 (74.6)	1.29	1,573 (14.7)	1,640 (15.3)	0.13
6-10	12,531 (40.5)	374,143 (18.0)		4,483 (41.8)	4,804 (44.8)	
≥11	12,245 (39.6)	155,991 (7.5)		4,670 (43.5)	4,283 (39.9)	
VACS Index score						
<20	117 (0.4)	38,767 (1.9)	0.80	39 (0.4)	35 (0.3)	0.03
20-34	7,748 (25.0)	1,035,820 (49.7)		3,151 (29.4)	3,033 (28.3)	
35-54	14,501 (46.9)	701,034 (33.7)		5,734 (53.5)	5,702 (53.2)	
≥55	6,393 (20.7)	55,826 (2.7)		961 (9.0)	1,224 (11.4)	
Missing	2,180 (7.1)	251,955 (12.1)		841 (7.8)	732 (6.8)	

Notes: all statistics reported as n (%); up to five unexposed individuals were matched to each exposed individual; *unexposed matches were weighted according to number of matches

Abbreviations: PS, propensity score; SMD, standardized mean difference; HCV, hepatitis C virus; AUD, alcohol use disorder; VACS, Veterans Aging Cohort Study

Table 3. Estimated average pre- and post-index date AUDIT-C scores and difference-in-differences (Diff-in-Diff), overall, by baseline AUDIT-C score, and by average daily dose of spironolactone

		Exposed	Unexposed
		***n* = 10,726**	***n* = 34,461**
All patients	**Pre**	3.07 (0.02)	2.96 (0.01)
	Post	2.16 (0.02)	2.22 (0.01)
	D^n	-0.91 (0.03)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.17 (-0.09, -0.25), $p < 0.0001$	
By baseline AUDIT-C score			
		***n* = 7,362**	***n* = 24,098**
1-3	**Pre**	1.64 (0.02)	1.62 (0.01)
	Post	1.46 (0.02)	1.52 (0.01)
	D^n	-0.18 (0.03)	-0.11 (0.02)
	Diff-in-Diff (95% CI)	-0.07 (-0.01, -0.14), $p = 0.0231$	
		***n* = 2,439**	***n* = 7,701**
4-7	**Pre**	4.85 (0.04)	4.83 (0.02)
	Post	3.29 (0.04)	3.39 (0.02)
	D^n	-1.56 (0.05)	-1.43 (0.03)
	Diff-in-Diff (95% CI)	-0.13 (-0.02, -0.24), $p = 0.0221$	
		***n* = 925**	***n* = 2,662**
≥8	**Pre**	9.72 (0.06)	9.70 (0.03)
	Post	4.68 (0.06)	5.13 (0.03)
	D^n	-5.04 (0.08)	-4.57 (0.05)
	Diff-in-Diff (95% CI)	-0.47 (-0.29, -0.66), $p < 0.0001$	
By average dose of spironolactone (mg/day)			
		***n* = 2,640**	***n* =34,461**
<25	**Pre**	3.00 (0.03)	2.96 (0.01)
	Post	2.16 (0.03)	2.22 (0.01)
	D^n	-0.84 (0.05)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.09 (0.01, -0.19), $p = 0.0658$	
		***n* = 6,110**	***n* = 34,461**
25-49	**Pre**	2.98 (0.04)	2.96 (0.01)
	Post	2.15 (0.04)	2.22 (0.01)
	D^n	-0.83 (0.06)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.08 (0.05, -0.21), $p = 0.2140$	
		***n* = 1,976**	***n* = 34,461**
≥50	**Pre**	3.61 (0.07)	2.96 (0.01)
	Post	2.17 (0.07)	2.22 (0.01)
	D^n	-1.44 (0.10)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.69 (-0.50, -0.89), $p < 0.0001$	

Notes: statistics reported as mean (standard error)

Abbreviations: AUDIT-C, Alcohol Use Disorders Identification Test - Consumption; Pre, pre-index AUDIT-C score; Post, post-index AUDIT-C score; D^n, change in AUDIT-C score; Diff-in-Diff, difference-in-difference; CI, confidence interval

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Figure Legends

Figure 1. Spironolactone decreased binge-like alcohol drinking in mice. (**A**) Spironolactone dose-dependently reduced alcohol intake (g/kg of body weight) in mice drinking a sweetened alcohol solution [20% alcohol (*v/v*), 3% glucose (*w/v*), and 0.1% saccharin (*w/v*)], and female mice drank significantly more alcohol than male mice. Males: $n = 8$; Females: $n = 7$. (**B**) Spironolactone dose-dependently reduced alcohol intake (g/kg of body weight) in mice drinking an unsweetened alcohol solution [20% (*v/v*)]. Males: $n = 8$; Females: $n = 7$. (**C**) Spironolactone had no effect on the intake (mL/kg of body weight) of a non-alcohol-containing sweetened solution [0.3% glucose (*w/v*) and 0.01% saccharin (*w/v*)] in mice. Males: $n = 8$; Females: $n = 7$. Separate cohorts of mice were used for each drinking solution. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, *vs.* vehicle; $^{\#}p < 0.05$, male *vs*. female. DID: drinking-in-the-dark.

Figure 2. Spironolactone did not affect motor coordination or spontaneous locomotion in mice. (**A**) Spironolactone treatment had no effect on motor coordination in mice that received a saline injection and were tested 30 min and 90 min later, on the rotarod. Males: $n = 6$; Females: $n = 5$. (**B**) Systemic administration of alcohol (1.5 g/kg) significantly impaired motor coordination in mice. Males: $n = 6$; Females: $n = 5$. Spironolactone treatment had no effect on alcohol-induced ataxia on the rotarod test at any time-point. $^{\#\#\#\#}p < 0.0001$, *vs*. Baseline. (**C**) Spironolactone had no effect on blood alcohol levels 30 min and 90 min after systemic administration of alcohol (1.5 g/kg). $^{\#\#\#\#}p < 0.0001$, 30 min *vs.* 90 min. Males: $n = 6$; Females: $n = 5$. **(D)** Spironolactone had no effect on spontaneous locomotion in the circular corridor. Males: $n = 8$; Females: $n = 6$.

Figure 3. Spironolactone decreased operant alcohol self-administration in alcohol-dependent **(DEP)** and nondependent **(NON)** male **and female** rats. (**A**) Spironolactone administration decreased alcohol self-

administration in nondependent and alcohol-dependent **male** rats tested under a fixed-ratio 1 schedule of reinforcement. ****$p < 0.0001$, *vs*. vehicle. $^{\#\#\#\#}$**$p < 0.0001$, *vs*. NON.** Nondependent: $n = 12$; Dependent: $n = 12$. (**B**) Alcohol-induced ataxia was higher in nondependent than dependent **male** rats; spironolactone did not affect alcohol-induced ataxia in either group. **$p < 0.01$, difference between dependent and nondependent **male** rats. Nondependent: $n = 15$; Dependent: $n = 9$. (**C**) Spironolactone had no effect on blood alcohol levels 30 min, 60 min, 120 min, and 180 min after systemic administration of alcohol (1.5 g/kg). Nondependent: $n = 15$; Dependent: $n = 9$. **(D) Spironolactone administration decreased alcohol self-administration in nondependent and alcohol-dependent female rats tested under a fixed-ratio 1 schedule of reinforcement. *$p < 0.05$, ****$p < 0.0001$, *vs*. vehicle. $^{\#\#\#\#}p < 0.0001$, *vs*. NON. Nondependent: $n = 7$; Dependent: $n = 8$.**

Figure 4. Difference-in-difference estimates and 95% confidence intervals of self-reported changes in Alcohol Use Disorders Identification Test-Consumption-C (AUDIT-C) scores associated with spironolactone exposure, overall and by baseline AUDIT-C score and average daily dose of spironolactone. Difference-in-differences = reported AUDIT-C decrease among spironolactone-exposed individuals minus reported AUDIT-C decrease among propensity-score matched unexposed controls **during the study period**. *$p < 0.05$, ****$p < 0.0001$, *NS:* not significant.

Figure 1

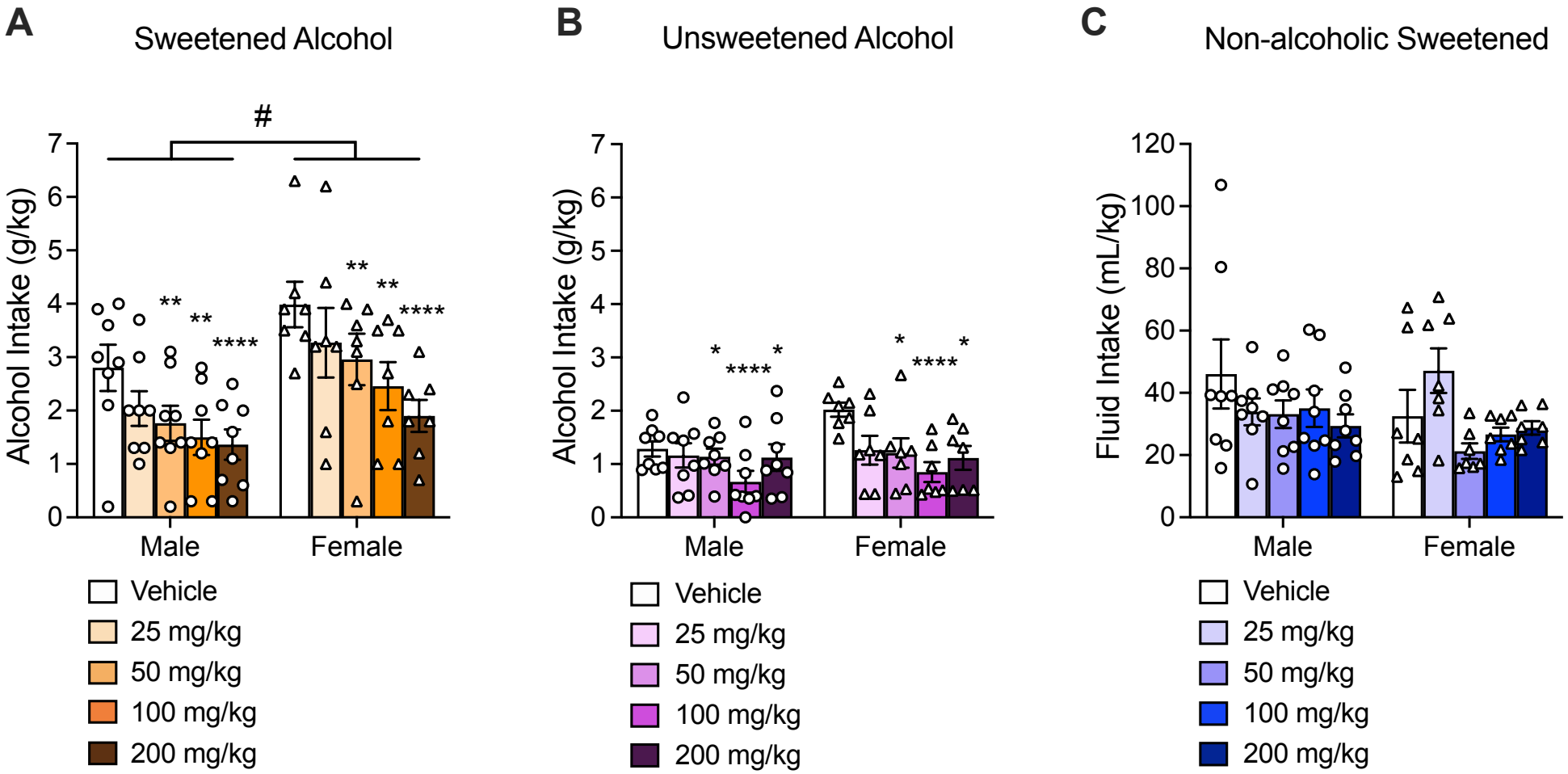

A
Sweetened Alcohol
B
Unsweetened Alcohol
C
Non-alcoholic Sweetened
Alcohol Intake (g/kg)
Fluid Intake (mL/kg)
Male
Female
Vehicle
25 mg/kg
50 mg/kg
100 mg/kg
200 mg/kg

Figure 2

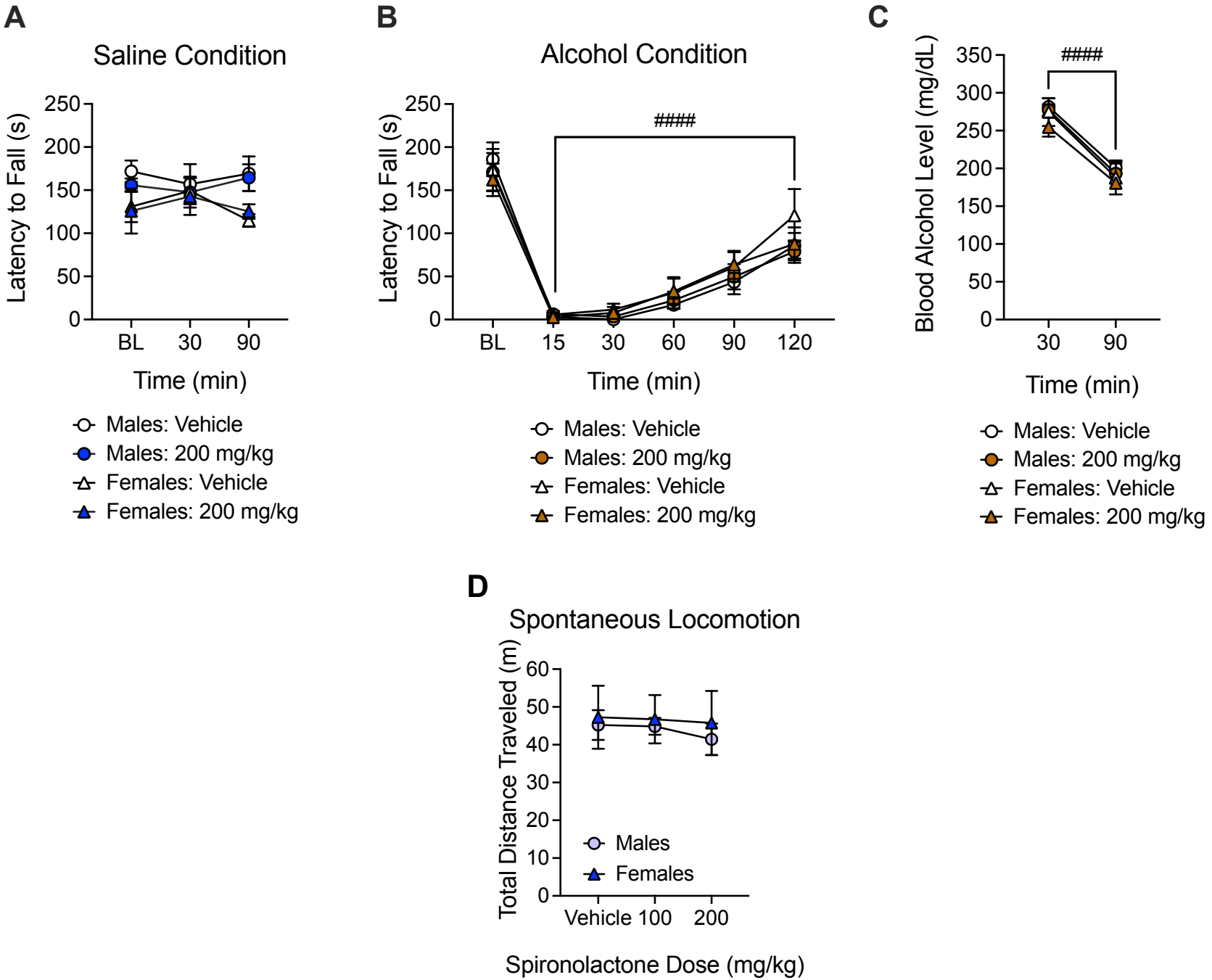

A
Saline Condition
Latency to Fall (s)
250
200
150
100
50
0
BL
30
90
Time (min)
Males: Vehicle
Males: 200 mg/kg
Females: Vehicle
Females: 200 mg/kg
B
Alcohol Condition
Latency to Fall (s)
####
BL
15
30
60
90
120
Time (min)
Males: Vehicle
Males: 200 mg/kg
Females: Vehicle
Females: 200 mg/kg
C
Blood Alcohol Level (mg/dL)
350
300
250
200
150
100
50
0
####
30
90
Time (min)
Males: Vehicle
Males: 200 mg/kg
Females: Vehicle
Females: 200 mg/kg
D
Spontaneous Locomotion
Total Distance Traveled (m)
60
50
40
30
20
10
0
Males
Females
Vehicle
100
200
Spironolactone Dose (mg/kg)

Figure 3

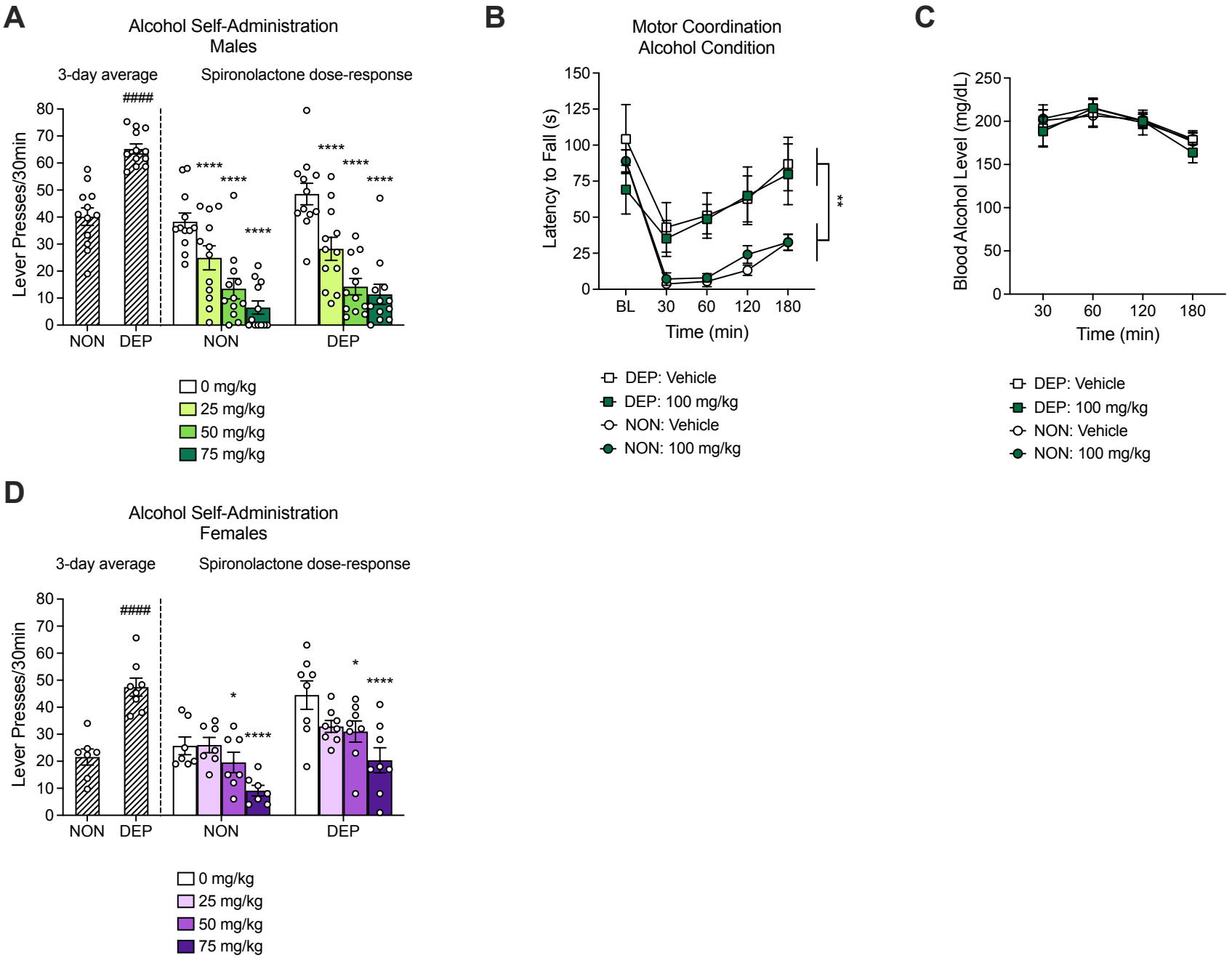
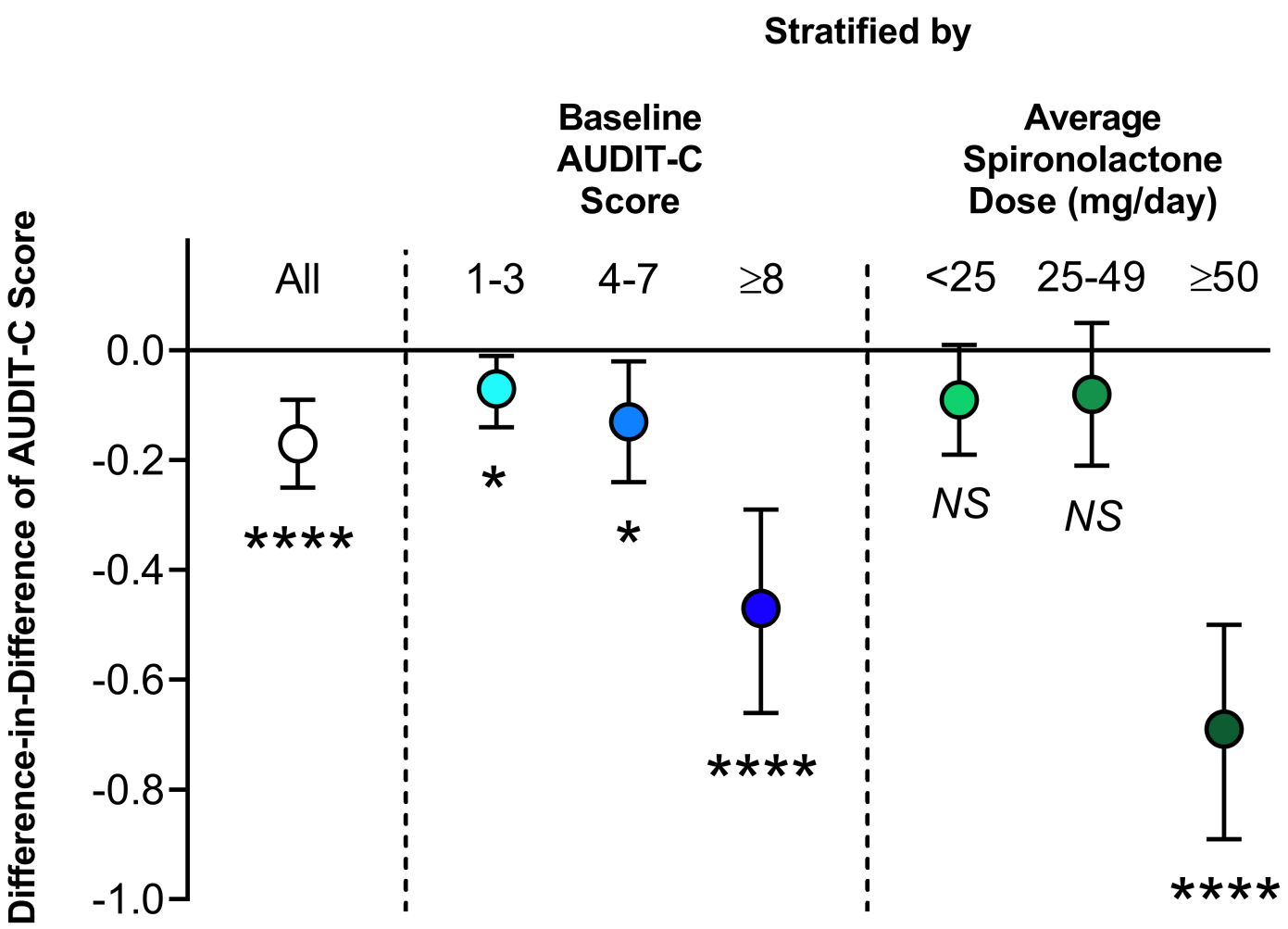

A
Alcohol Self-Administration
Males
3-day average
Spironolactone dose-response
Lever Presses/30min
NON
DEP
NON
DEP
0 mg/kg
25 mg/kg
50 mg/kg
75 mg/kg
B
Motor Coordination
Alcohol Condition
Latency to Fall (s)
BL
30
60
120
180
Time (min)
DEP: Vehicle
DEP: 100 mg/kg
NON: Vehicle
NON: 100 mg/kg
C
Blood Alcohol Level (mg/dL)
30
60
120
180
Time (min)
DEP: Vehicle
DEP: 100 mg/kg
NON: Vehicle
NON: 100 mg/kg
D
Alcohol Self-Administration
Females
3-day average
Spironolactone dose-response
Lever Presses/30min
NON
DEP
NON
DEP
0 mg/kg
25 mg/kg
50 mg/kg
75 mg/kg

Figure 4


Stratified by
Baseline AUDIT-C Score
Average Spironolactone Dose (mg/day)
All
1-3
4-7
≥8
<25
25-49
≥50
Difference-in-Difference of AUDIT-C Score
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
****
*
*
****
NS
NS
****

Supplementary Information

Farokhnia M, Rentsch CT, Chuong V, McGinn MA, Elvig SK, Douglass EA, Gonzalez LA, Sanfilippo JE, Marchette RCN, Tunstall BJ, Fiellin DA, Koob GA, Justice AC, Leggio L, Vendruscolo LF. Spironolactone as a Potential New Pharmacotherapy for Alcohol Use Disorder: Convergent Evidence from Rodent and Human Studies.

Table of Contents:

Appendix 1. Spironolactone and alcohol use in rodents: Psychopharmacology studies (methods)

1.1. Animals

Adult, male (*n* = 24) and female (*n* = 21) C57BL/6J mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were individually housed in standard cages. Adult, male (*n* = 48) and female (*n* = 15) Wistar rats were acquired from Charles River (Raleigh, NC, USA). Rats were group-housed 2-3 per cage. Mice and rats were held in temperature- and humidity-controlled rooms (different rooms for rats and mice) with a reverse 12 h/12 h light/dark cycle (lights off at 7:00 AM) and had *ad libitum* access to food and water, except during behavioral testing. Mice and rats were at least 8 weeks old at the beginning of the experiments. Behavioral tests were conducted during the dark cycle. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program.

1.2. Drugs

Spironolactone (Sigma Aldrich, St. Louis, MO, USA – catalog # S3378) was mixed in Tween 80 (4% *v/v*; Fisher Chemical, Fairlawn, NJ, USA) and diluted with 0.9% saline. Mice were subcutaneously (s.c.) injected with vehicle (4% Tween 80, *v/v*, in saline) or spironolactone (25 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg; volume of injection: 10 ml/kg) 30 min before alcohol drinking in a within-subjects, Latin-square design. Mice were s.c. injected with vehicle (4% Tween 80, *v/v*, in saline) or spironolactone (100 mg/kg and 200 mg/kg; volume of injection: 10 ml/kg) for circular corridor testing. A cohort of dependent and nondependent male rats were s.c. injected with vehicle or spironolactone (100 mg/kg; volume of injection: 1 ml/kg) for rotarod testing and blood alcohol level measurements. Because we observed skin irritation following s.c. administration, we switched to intraperitoneally (i.p.) for subsequent experiments.

Mice were i.p. injected with vehicle (4% Tween 80, *v/v*, in saline) or spironolactone (200 mg/kg; volume of injection: 10 ml/kg) for rotarod testing and blood alcohol level measurements. For rat alcohol drinking studies, another male and female cohort of rats were i.p. injected with vehicle (4% Tween 80, *v/v*, in saline) or spironolactone (25 mg/kg, 50 mg/kg, and 75 mg/kg; volume of injection: 1 ml/kg) 60 min before alcohol drinking in a within-subjects, Latin-square design. Doses of spironolactone tested on mice and rats were selected based on previous studies and body surface area of species [1-3]. The alcohol used for i.p. injections in mice and rats was prepared using 190 proof ethanol (Pharmco, Shelbyville KY, USA) diluted with 0.9% saline to produce a 20% (*v/v*) solution. The volume of injection was adjusted according to the animal's body weight.

1.3. Drinking solutions

All drinking solutions were prepared with 190 proof ethanol (The Warner-Graham Company, Cockeysville, MD, USA) and tap water. For mice, the unsweetened alcohol solution was 20% (*v/v*). The sweetened alcohol solution was prepared with 20% alcohol (*v/v*), 3% glucose (*w/v*), and 0.1% saccharin (*w/v*) [4]. The non-alcohol-containing sweet solution was prepared with 0.3% glucose (*w/v*) and 0.01% saccharin (*w/v*), to produce drinking (mL) levels closer to what mice drank with alcohol containing solutions. For rats, the unsweetened alcohol solution was prepared with 190 proof ethanol diluted with tap water to 10% (*w/v*).

1.4. Drinking-in-the-dark test in mice

A drinking-in-the-dark (DID) test was used to evaluate binge-like alcohol drinking in mice [5]. A 4-day protocol was used, in which mice had access to either unsweetened alcohol, sweetened alcohol, or a non-alcohol-containing sweet solution for 2 h during the first 3 days, and for 4 h on the 4th day. Different cohorts of male and female mice were used to test the intake of unsweetened alcohol, sweetened alcohol, or non-alcohol-containing sweet solutions. Mice were then given 3 consecutive days off from DID. During a DID

session, food was removed from the home-cage, and water was replaced with one of the drinking solutions 3 h into the dark phase [5]. This schedule was used for three weeks before switching to a modified DID schedule to test the effects of spironolactone. For the modified DID schedule, mice were allowed access to the drinking solutions for 2 h on day 1 and 4 h on day 2. This was followed by at least 1 day off from DID and then the same schedule was repeated (i.e., 2 h on day 1 and 4 h on day 2). The effects of spironolactone (two doses per week) were evaluated during the 4 h DID session. Spironolactone and vehicle were administered s.c. 30 min prior to drinking solution access. Drinking bottles with alcohol and non-alcohol-containing solutions were weighed before and after each DID session, and the change in weight from these measurements was used to calculate the amount of alcohol intake in g/kg of body weight and the intake of a non-alcohol-containing solution in mL/kg of body weight.

1.5. Food and water intake measurements in mice

Mice that were previously given access to the sweetened alcohol solution during the DID test were treated with either vehicle or 200 mg/kg of spironolactone, and food and water were measured at 6 h and 24 h after treatment. The dose of 200 mg/kg was selected for this experiment and the next experiment, because it was the highest dose tested in the DID experiment described above.

1.6. Rotarod test and blood alcohol level measurements in mice

The effects of spironolactone on alcohol-induced ataxia were evaluated using an accelerating rotarod test [6] in mice that were drinking the sweetened alcohol solution. Mice were first habituated on the rotarod apparatus (Rotamex-5; Columbus Instruments, Columbus OH) with the rod set at a constant speed of 4 rpm. However, during training trials and testing trials, the rod was set to accelerate at a constant rate of 8 rpm/min from 4 rpm up to 40 rpm. The latency to fall was detected and recorded by infrared beam sensors. The maximum cutoff latency was set at 5 min, when mice were removed from the rod. Mice first underwent 5

consecutive training trials on the rotarod apparatus, separated by 5 min rest intervals. All mice were given a minimum resting period of 24 h following completion of rotarod training. On the testing day, mice received 2 baseline trials, separated by 5 min rest intervals. We tested the effects of spironolactone *per se* on motor coordination. Mice were treated with either 200 mg/kg spironolactone or vehicle (a two-day, counterbalanced, within-subjects design) and i.p. injected with 0.9% saline 30 min later. Mice were then tested on the rotarod 30 min after the saline injection. Mice that received i.p. saline were tested on the rotarod at 30 min and 90 min after saline injection. The effects of spironolactone on alcohol-induced ataxia were then tested in a separate rotarod session. Mice were injected i.p. with spironolactone or vehicle and then injected with 1.5 g/kg of alcohol 30 min later. Mice were returned to their home-cages for 30 min. Mice that received i.p. alcohol were tested on the rotarod at 15 min, 30 min, 60 min, 90 min, and 120 min after alcohol injection, and blood was collected via the submandibular facial vein immediately following the 30 min and 90 min rotarod trials to measure blood alcohol levels. Blood serum was separated by centrifugation and analyzed using an Analox Alcohol Analyzer instrument (Analox Technologies North America, Toronto, Ontario, Canada).

1.7. Circular corridor test in mice

The effects of spironolactone on spontaneous locomotion were assessed using a circular corridor test on mice from the cohort that had previously received sweetened alcohol. Mice were placed in a circular corridor apparatus (Thermal Gradient Ring, Ugo Basile, Gemonio, Italy) at room temperature (22°C). Mice were first habituated to the circular apparatus for 15 min. After a minimum of 24 h, the mice were s.c. administered either vehicle or spironolactone (100 mg/kg, 200 mg/kg) and returned to their home-cages. After 30 min, the mice were allowed to freely move in the circular corridor for 15 min. ANY-maze Video Tracking Software (Stoelting Co., Wood Dale, IL, USA) was used to track the total distance traveled (m) of each mouse.

1.8. Operant alcohol self-administration in rats

Self-administration sessions were conducted in standard operant self-administration chambers (Med Associates, St. Albans, VT, USA). The rats were trained to self-administer 10% (w/v) alcohol and water under a fixed-ratio 1 (FR1) schedule of reinforcement, in which each operant response on the alcohol lever or water lever was reinforced with 0.1 ml of solution, as previously described [7]. A cue light located above the alcohol-associated lever was illuminated following a response on the alcohol-associated lever for the duration of alcohol delivery (2 sec). After 6 daily 30-min training sessions, the rats were split into two groups that were matched by the average number of lever presses for alcohol in the last three training sessions. One group was exposed to alcohol vapor (dependent) and the other group was exposed to air without alcohol (nondependent).

1.9. Alcohol vapor exposure in rats

Male and female rats were made alcohol-dependent by chronic, intermittent alcohol vapor exposure, as previously described [7]. They were exposed to daily cycles of 14 h of alcohol vapor, followed by 10 h of room air, for a minimum of 4 weeks. The target blood alcohol levels during alcohol vapor exposure ranged between 150 and 250 mg/dl. Behavioral testing occurred in 2-3 30-min sessions per week during acute spontaneous withdrawal, i.e., 6-8 h after the alcohol vapor exposure ended. Nondependent rats were not exposed to alcohol vapor but were tested at the same time as the dependent rats for operant alcohol self-administration. Spironolactone was tested under an FR1 schedule of reinforcement.

1.10. Rotarod test and blood alcohol level measurements in male rats

A separate cohort of alcohol-dependent (vapor exposed) and nondependent male rats were tested on the rotarod. The training and testing procedures for the rotarod test were identical to the procedures for mice described above, except for the following: across two testing days, rats were treated s.c. with either 100

mg/kg of spironolactone or vehicle in a within-subjects design. Thirty minutes later, all rats were injected i.p. with 1.5 g/kg of alcohol. The rats were tested on the rotarod at 30 min, 60 min, 120 min, and 180 min after the alcohol injection. Blood was collected via the tail vein immediately following the 30 min, 60 min, 120 min, and 180 min rotarod trials to measure blood alcohol levels, as described above.

1.11. Statistical analysis

The drinking data in mice were examined using a two-way repeated measures analysis of variance (ANOVA), with dose as the within-subjects factor and sex as the between-subjects factor. Data from the rotarod test and blood alcohol levels were analyzed using a three-way repeated measures ANOVA, with dose and time (min) as within-subjects factors and sex as the between-subjects factor. Saline- and alcohol-treated conditions for the rotarod test were analyzed separately. The total distance traveled (m) by mice on the circular corridor was analyzed using a two-way repeated measures ANOVA, with dose as the within-subjects factor and sex as the between-subjects factor. Food and water intake data were analyzed using a three-way repeated measures ANOVA with dose and time as the within-subjects factors and sex as the between-subjects factor. The operant alcohol self-administration data in rats comparing the number of lever presses averaged across three operant sessions were analyzed with an unpaired Student's t-test. The operant alcohol self-administration data evaluating the effect of spironolactone on alcohol intake in rats were analyzed with a two-way repeated-measures ANOVA with dose as the within-subjects factor and group (dependent and nondependent) as the between-subjects factor. The rotarod test data and blood alcohol levels in rats were analyzed using a three-way repeated measures ANOVA, with dose and time as within-subjects factors and group (dependent and nondependent) as the between-subjects factor. *Post hoc* comparisons were performed, when appropriate, using the Dunnett's test following two-way repeated measures ANOVAs and the Holm-Sidak multiple comparisons test following three-way repeated measures ANOVAs. Grubb's test was used to identify significant outliers. All data are presented as mean and standard

error of the mean (SEM). Analyses were performed using GraphPad Prism 8 Software (San Diego, CA, USA). Statistical significance was considered at $p < 0.05$ (two-sided) for all analyses.

Appendix 2. Spironolactone and alcohol use in humans: A pharmacoepidemiology study (methods)

2.1. Study design and population

An observational cohort study was conducted, using electronic health record data from the US Department of Veterans Affairs (VA), which is the largest integrated healthcare system in the US and comprises more than 1,200 points of care nationwide, including hospitals, medical centers, and community outpatient clinics. Available data include information on all outpatient and inpatient encounters, including demographics, diagnoses, pharmacy dispensing records, laboratory measures, and routinely collected measurement of smoking and alcohol consumption. We extracted data on all patients born between 1945 and 1965 who had at least one outpatient visit on or after 1 October 1999, which included approximately half of all patients in care. This study was approved by the Institutional Review Boards of Yale University (ref #1506016006) and VA Connecticut Healthcare System (ref #AJ0013), granted a waiver of informed consent, and deemed Health Insurance Portability and Accountability Act compliant.

2.2. Exposure groups

Pharmacy records were extracted to identify patients who did and did not receive spironolactone dispensed at VA pharmacies. We identified the first prescription for spironolactone during the study period and required a 180-day washout period to have new episodes of spironolactone exposure. Exposure to spironolactone was defined as receipt of two or more spironolactone fills for at least 60 continuous days, for any indication, between 1 January 2009 and 30 September 2015, from the following outpatient clinics: primary care, general internal medicine, cardiology, gastroenterology, hepatology, nephrology (excluding dialysis clinics), women's clinic, and endocrinology. These clinics were chosen because they were the source of most (>80%) of the spironolactone prescriptions in the VA. For unexposed comparators, we selected patients who attended at least one of these clinics, but never received spironolactone, to ensure that

unexposed patients came from the same source population, were exposed to an overall similar medical care, and had an equal opportunity to receive spironolactone.

We created an "index date" (also referred to as "baseline"), which was defined as the first fill date for spironolactone exposed patients and a randomly selected outpatient visit date for unexposed patients. We excluded patients with no outpatient care in the year prior to their index date due to the inability to capture baseline characteristics. Patients with no measurement of alcohol consumption in the two years prior to their index date and those who reported no alcohol consumption based on the closest measurement to baseline were also excluded.

2.3. Propensity score model and matching

To balance the distribution of all potential confounders across treatment groups, we performed propensity score matching. This matching was done by first modelling the probability (i.e., propensity) of receiving spironolactone as a function of measured covariates that are associated with spironolactone receipt and/or changes in alcohol consumption [8, 9]. There were 72 variables used in the propensity score model, including: year of index date, age at baseline, race/ethnicity, sex, smoking status, body mass index at baseline, site prescribing pattern (defined as the prevalence of spironolactone use by site and year), laboratory values closest to the index date, diabetes complications severity index [10] at baseline, history of pain diagnoses prior to baseline (including neuropathy, osteoarthritis, or pain in the abdomen, back, chest, extremity, or neck, headache, or fracture), history of medical or psychiatric conditions prior to baseline (including atrial fibrillation, myocardial infarction/coronary artery disease, peripheral vascular disease, diabetes, nephrolithiasis, glomerulonephritis, hyperlipidemia, pancreatitis, hepatitis C infection, hepatitis B infection, hepatic decompensation, seizure, cancer, substance use disorder, post-traumatic stress disorder, major or other depression, anxiety, bipolar disorder, schizophrenia, or schizoaffective disorder), and the Veterans Aging Cohort Study (VACS) Index. The VACS Index is a validated composite measure of physiologic injury incorporating age, hemoglobin, fibrosis-4 score, estimated glomerular filtration rate,

CD4 count, HIV-1 RNA, and HCV status [11]. We also included variables that captured attendance to clinics (including primary care, diabetic retinal screening, rheumatology, infectious disease, nephrology, neurology, pain, allergy, chiropractic, dental, diabetes, emergency department, electrocardiogram lab, ophthalmology, hematology, oncology, homeless program, nutrition, orthopedics, substance use, mental health, or post-traumatic stress disorder), frequency of all-cause hospitalizations, and the total number of unique clinics visited in the year prior to baseline. Lastly, variables denoting total number of chronic medications and binary indicators for receipt of specific medication classes at baseline (including antipsychotics, non-steroidal anti-inflammatory drugs, opioids, muscle relaxants, and antidepressants) were included in the model. Interaction terms were explored for significance, and five were kept in the final model (all p's < 0.05).

We hypothesized that medication effects on alcohol consumption may vary based on differences among individual characteristics, including the presence, or not, of an AUD diagnosis and, among those with AUD, whether they were seeking treatment [12-15]. Therefore, propensity scores were estimated using separate multivariable logistic regression models for three mutually exclusive groups: no AUD at baseline, AUD at baseline without attendance at a substance use treatment program, and AUD at baseline with attendance at a substance use treatment program. Estimating propensity scores separately has been shown to be unbiased, particularly in subgroup analyses with small sample sizes [16-18]. The C-statistic for each model was 0.85, 0.87, and 0.88, respectively, indicating adequate discrimination between spironolactone exposed and unexposed individuals [19].

Exposed individuals were then matched to unexposed individuals with equal propensity for exposure to the fourth or fifth decimal place, giving preference to the latter. We conducted propensity score matching within pre-specified subgroups of patients based on baseline AUD and substance use treatment program attendance, matching each spironolactone exposed patient to up to five unexposed patients in the same calendar year, using a greedy matching algorithm [20]. We then aggregated these matched subgroups to create the overall matched cohort [21].

2.4. Outcome and follow-up

Alcohol consumption was assessed using Alcohol Use Disorders Identification Test-Consumption (AUDIT-C), a three-question self-reported alcohol screening questionnaire that detects heavy drinking and/or active AUD [22, 23]. AUDIT-C scores range from 0-12 with the likelihood of physiologic injury and mortality increasing as AUDIT-C scores increase [24]. An AUDIT-C score of zero indicates no current alcohol use, 1-3 suggests low-risk drinking, 4-7 suggests at-risk drinking, and ≥8 suggests hazardous or heavy episodic alcohol consumption. Since 2008, the VA has required annual AUDIT-C screening for all patients in primary care [25].

Patients were followed for a maximum of two years from their index date or until their last VA visit, death, or 30 September 2015. Additionally, spironolactone exposed patients were censored at 30 days after the end of their last spironolactone prescription (allowing for gaps between fills up to 50% of the index prescription duration). To ensure equal follow-up time within matched sets, unexposed individuals were censored at the total follow-up time of their matched exposed patient. Although evidence of alcohol consumption at baseline, as measured by the AUDIT-C, was a criterion for study inclusion, we did not restrict matching eligibility on the availability of a follow-up AUDIT-C (the outcome), as such a restriction would not be available in an analogous randomized clinical trial. If an exposed patient did not have a follow-up AUDIT-C, we removed their entire matched set of unexposed patients to maintain a balanced sample. If an unexposed patient did not have a follow-up AUDIT-C, we kept the remaining patients in their matched set in the analytic sample, as long as there was another unexposed patient in the set.

2.5. Statistical analysis

We calculated absolute standardized mean differences to examine balance between exposed and unexposed patients included in the full cohort and the final analytic cohort after propensity score matching, and

considered ≤0.2 as balanced [26]. Among those in the matched cohort, we calculated the average pre- and post-index AUDIT-C scores. Pre-index AUDIT-C scores were defined as the closest on or before the index date, within a maximum of two years. Post-index AUDIT-C scores were defined as the closest measure to the end of exposure or within 30 days of end of follow-up. We then used multivariable difference-in-difference (Diff-in-Diff) linear regression models [27, 28] to estimate the differential change between pre- and post-index AUDIT-C scores among exposed and unexposed patients. We also performed subgroup analyses stratified by self-reported level of alcohol consumption at baseline (as determined by AUDIT-C) and average daily dose of spironolactone during follow-up. Daily dose was categorized as <25, 25-49, and ≥50 mg/day. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

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