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## Spironolactone as a potential new pharmacotherapy for alcohol use disorder: convergent evidence from rodent and human studies

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### AUTHOR CONTRIBUTIONS

MF and LL conceptualized the study. MF, CTR, VC, MAM, RCNM, BJT, GFK, ACJ, LL, and LFV designed the study and developed the data analysis plan. For the rodent studies, VC, MAM, SKE, EAD, and LAG conducted the experiments and RCNM, BJT, GFK, and LFV provided support. For the pharmacoepidemiology study, CTR managed and analyzed data and DAF and ACJ provided support. MF, CTR, VC, and MAM wrote the first draft of the manuscript and JES, LL, and LFV contributed to the first draft of the manuscript. All authors reviewed, provided feedback, and approved the final manuscript. GFK, ACJ, and LL provided funding for this study.

### COMPETING INTERESTS

The authors declare no competing interests.

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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 sweet solution without alcohol, 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.

## 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.

Spirolactone is an FDA-approved MR antagonist medication used to treat essential hypertension, heart failure, edema, primary hyperaldosteronism, and hypokalemia. Spirolactone 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 et al. 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 et al. 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.

## METHODS AND MATERIALS

### **Spironolactone and alcohol use in rodents: psychopharmacology studies**

Full methodological details are described in Appendix 1. Briefly, adult male and female C57BL/6 J 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].

### **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.

## RESULTS

### Effects of spironolactone in rodents

#### 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 (Fig. 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 ( $p = 0.02$ ; Fig. 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 reduced non-alcohol-containing sweet solution intake, compared to the vehicle condition. Furthermore, the ANOVA did not show a sex effect ( $F_{1,13} = 0.66$ ,  $p = 0.42$ ) nor 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 (Fig. 1C).

**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).

**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 that 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 (Fig. 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 that 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 ameliorated 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 (Fig. 2B).

Immediately following the rotarod trials at 30 min and 90 min, blood was collected for blood alcohol levels measurement. 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 (Fig. 2C). No main effect of sex on alcohol-induced ataxia ( $F_{1,9} = 0.31, p = 0.58$ ; Fig. 2B) or blood alcohol levels ( $F_{1,9} = 1.33, p = 0.27$ ; Fig. 2C) was found.

**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  $\times$  sex interaction ( $F_{2,24} = 0.18, p = 0.83$ ; Fig. 2D).

**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

(Fig. 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  $\times$  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 (Fig. 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 (Fig. 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 (Fig. 3D). The ANOVA did not show a significant interaction between spironolactone and alcohol dependence ( $F_{3,39} = 0.99, p = 0.41$ ).

### Effects of spironolactone receipt in humans

**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, 3016 (28.1%) were matched to five unexposed individuals, 2287 (21.3%) to four, 1541 (14.4%) to three, 1728 (16.1%) to two, and 2154 (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  $\leq 0.2$ , with most  $\leq 0.1$ ) [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  $\geq 50$  mg/day, respectively. Median follow-up time was 542 days (IQR 337–730 days).

**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  $\geq 8$ , 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  $\geq 50$  mg/day of spironolactone (Diff-in-Diff:  $-0.69$  points, 95% CI:  $-0.50$ ,  $-0.89$ ;  $p < 0.0001$ ) (Fig. 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, and this was not influenced 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 also 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 [44], 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 [45]. 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 expected 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 [46]. 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 [47]. 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 [48]; 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 [49] and mortality [50]. 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 [51], anxiety, obsessive craving [18, 19], and alcohol drinking [19] in patients with AUD. Primary aldosteronism in humans was associated with increased anxiety [52, 53], and chronic treatment with aldosterone increased anxiety-like behavior in rats [54]. Many drugs that have shown promise in treating AUD (e.g., baclofen, gabapentin, pregabalin) also have anxiolytic effects [2, 55], and it is conceivable that at least part of spironolactone's effect on alcohol use may be driven by anxiety reduction [56-59]. Moreover, in adrenalectomized rats, aldosterone increased corticotropin-releasing factor (CRF) mRNA levels in the paraventricular nucleus of the hypothalamus and in the CeA [60]. Increased CRF activity in limbic areas, particularly the amygdala, drives negative

emotional states and drinking associated with alcohol dependence in rodent models [44, 45, 61, 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].

There is also evidence that enhanced 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 with 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 is unknown. 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 nonselective 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  $\alpha_1$ -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 effects, 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 examined 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 prospective 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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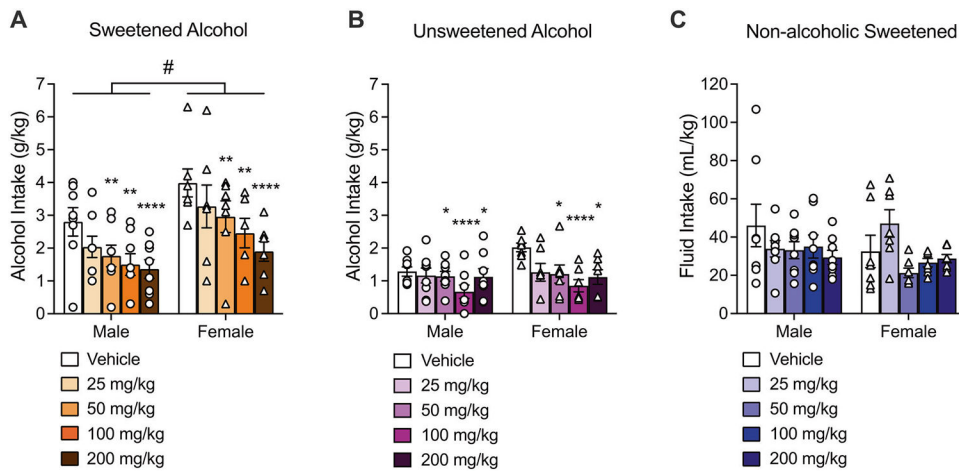
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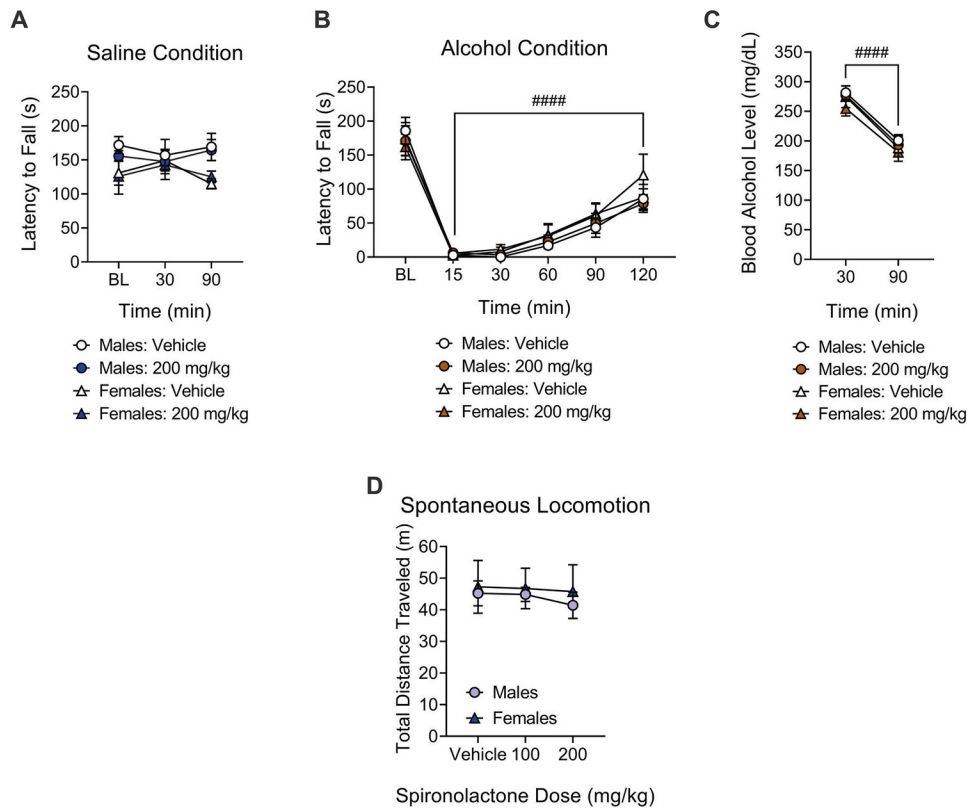
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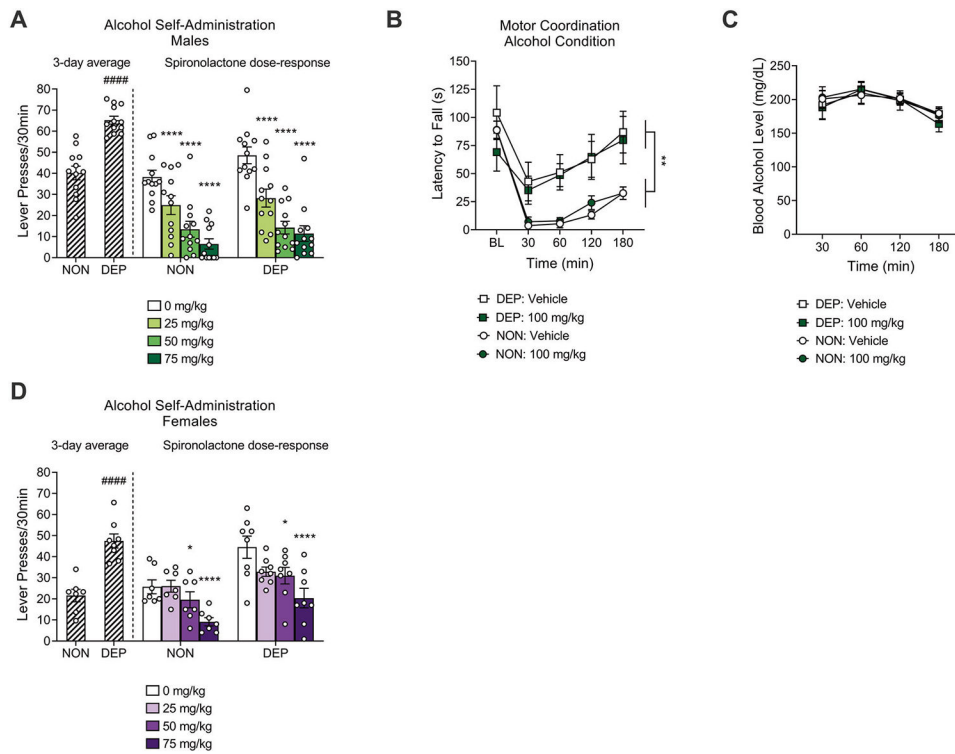
**Fig. 1. Spironolactone decreased binge-like alcohol drinking in mice.**

**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 sweet solution without alcohol [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.



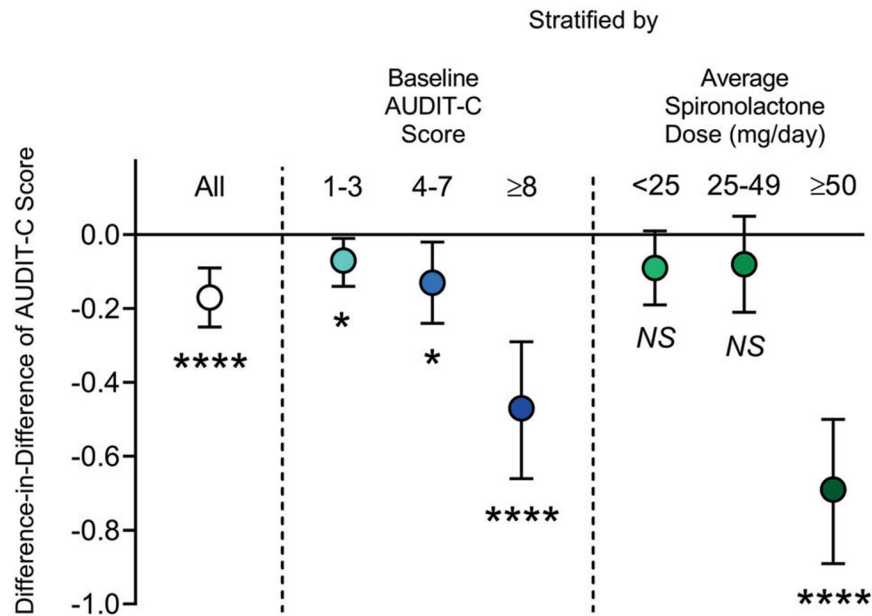
**Fig. 2. Spironolactone did not affect motor coordination or spontaneous locomotion in mice.**

**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:  $n = 6$ .



**Fig. 3. Spironolactone decreased operant alcohol self-administration in alcohol-dependent (DEP) and nondependent (NON) 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, 60, 120, and 180 min after systemic administration of alcohol (1.5 g/kg) in male rats. 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:  $n = 8$ .



**Fig. 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, by baseline AUDIT-C score, and by 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.

**Table 1.**

Spirolonactone did not affect (A) chow or (B) water intake in mice.

		Vehicle-treated				Spirolonactone-treated			
		Males		Females		Males		Females	
A	Total chow <sup>**</sup> 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 <sup>#####</sup>	2.51	0.343	4.00 <sup>†</sup>	0.259	2.11	0.404	4.00 <sup>†</sup>	0.886
B	Total water <sup>*</sup> 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 <sup>#####</sup>	1.69	0.164	3.16 <sup>††</sup>	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).

<sup>†</sup> *p* < 0.05, male vehicle-treated 24 h vs. female vehicle-treated 24 h and male spironolactone-treated 24 h vs. female-spirolonactone treated 24 h

<sup>††</sup> *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).

Distribution of baseline characteristics in spironolactone-exposed and unexposed individuals before and after propensity score matching.

**Table 2.**

Characteristic	Full cohort		Matched cohort		SMD
	Exposed n = 30,939	Unexposed n = 2,083,402	Exposed n = 10,726	Unexposed n = 10,726 <sup>a</sup>	
Age (years)					
<55	5389 (17.4)	549,459 (26.4)	1683 (15.7)	1716 (16.0)	0.03
55–59	6747 (21.8)	425,267 (20.4)	2059 (19.2)	2171 (20.2)	
60–64	12,016 (38.8)	682,783 (32.8)	4227 (39.4)	4198 (39.1)	
65	6787 (21.9)	425,893 (20.4)	2757 (25.7)	2641 (24.6)	
Race/ethnicity					
White	20,573 (66.5)	1,413,221 (67.8)	7317 (68.2)	7195 (67.1)	0.06
Black	7136 (23.1)	391,367 (18.8)	2401 (22.4)	2459 (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	1211 (3.9)	108,528 (5.2)	398 (3.7)	361 (3.4)	
Male sex	29,540 (95.5)	1,958,068 (94.0)	10,229 (95.4)	10,177 (94.9)	0.03
HCV+	4822 (15.6)	119,706 (5.8)	979 (9.1)	1290 (12.0)	0.06
AUD					
Never	20,398 (65.9)	1,676,893 (80.5)	7503 (70.0)	7417 (69.2)	0.07
Lifetime	3162 (10.2)	187,489 (9.0)	1202 (11.2)	1389 (13.0)	
Current	7379 (23.9)	219,020 (10.5)	2021 (18.8)	1920 (17.9)	
Substance use treatment program visit	9108 (29.4)	323,974 (15.6)	2095 (19.5)	1912 (17.8)	0.05
Any hospitalization	9976 (32.2)	169,306 (8.1)	2873 (26.8)	2280 (21.3)	0.18
Coronary artery disease	11,743 (38.0)	281,078 (13.5)	4673 (43.6)	3983 (37.1)	0.16
Diabetes	12,255 (39.6)	444,048 (21.3)	4793 (44.7)	4631 (43.2)	0.04
Hepatic decompensation	4515 (14.6)	5492 (0.3)	201 (1.9)	209 (1.9)	0.06
Hyperlipidemia	19,366 (62.6)	1,139,384 (54.7)	7676 (71.6)	7181 (67.0)	0.08
Abdominal pain	11,201 (36.2)	512,747 (24.6)	3693 (34.4)	3588 (33.5)	0.04
Chest pain	12,017 (38.8)	464,716 (22.3)	4498 (41.9)	4015 (37.4)	0.11
Any chronic pain	26,863 (86.8)	1,722,836 (82.7)	9466 (88.3)	9590 (89.4)	0.03
Number of medications					



Characteristic	Full cohort		Matched cohort		SMD	SMD
	Exposed <i>n</i> = 30,939	Unexposed <i>n</i> = 2,083,402	Exposed <i>n</i> = 10,726	Unexposed <i>n</i> = 10,726 <sup>a</sup>		
5	6163 (19.9)	1,553,268 (74.6)	1573 (14.7)	1640 (15.3)	1.29	0.13
6–10	12,531 (40.5)	374,143 (18.0)	4483 (41.8)	4804 (44.8)		
11	12,245 (39.6)	155,991 (7.5)	4670 (43.5)	4283 (39.9)		
VACS Index score						
<20	117 (0.4)	38,767 (1.9)	39 (0.4)	35 (0.3)	0.80	0.03
20–34	7748 (25.0)	1,035,820 (49.7)	3151 (29.4)	3033 (28.3)		
35–54	14,501 (46.9)	701,034 (33.7)	5734 (53.5)	5702 (53.2)		
55	6393 (20.7)	55,826 (2.7)	961 (9.0)	1224 (11.4)		
Missing	2180 (7.1)	251,955 (12.1)	841 (7.8)	732 (6.8)		

All statistics reported as *n* (%); up to five unexposed individuals were matched to each exposed individual.

SMD standardized mean difference, HCV hepatitis C virus, AUD alcohol use disorder, VACS Veterans Aging Cohort Study.

<sup>a</sup>Unexposed matches were weighted according to the number of matches.

**Table 3.**

Estimated average pre- and post-index date AUDIT-C scores and Diff-in-Diff, overall, by baseline AUDIT-C score, and by average daily dose of spironolactone.

		<b>Exposed</b>	<b>Unexposed</b>
		<b>n = 10,726</b>	<b>n = 34,461</b>
All patients	Pre	3.07 (0.02)	2.96 (0.01)
	Post	2.16 (0.02)	2.22 (0.01)
	D <sup>n</sup>	-0.91 (0.03)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.17 (-0.09, -0.25), <i>p</i> < 0.0001	
<b>By baseline AUDIT-C score</b>			
<b>1-3</b>		<b>n = 7362</b>	<b>n = 24,098</b>
	Pre	1.64 (0.02)	1.62 (0.01)
	Post	1.46 (0.02)	1.52 (0.01)
	D <sup>n</sup>	-0.18 (0.03)	-0.11 (0.02)
	Diff-in-Diff (95% CI)	-0.07 (-0.01, -0.14), <i>p</i> = 0.0231	
<b>4-7</b>		<b>n = 2439</b>	<b>n = 7701</b>
	Pre	4.85 (0.04)	4.83 (0.02)
	Post	3.29 (0.04)	3.39 (0.02)
	D <sup>n</sup>	-1.56 (0.05)	-1.43 (0.03)
	Diff-in-Diff (95% CI)	-0.13 (-0.02, -0.24), <i>p</i> = 0.0221	
<b>8</b>		<b>n = 925</b>	<b>n = 2662</b>
	Pre	9.72 (0.06)	9.70 (0.03)
	Post	4.68 (0.06)	5.13 (0.03)
	D <sup>n</sup>	-5.04 (0.08)	-4.57 (0.05)
	Diff-in-Diff (95% CI)	-0.47 (-0.29, -0.66), <i>p</i> < 0.0001	
<b>By average dose of spironolactone (mg/day)</b>			
<b>&lt;25</b>		<b>n = 2640</b>	<b>n = 34,461</b>
	Pre	3.00 (0.03)	2.96 (0.01)
	Post	2.16 (0.03)	2.22 (0.01)
	D <sup>n</sup>	-0.84 (0.05)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.09 (0.01, -0.19), <i>p</i> = 0.0658	
<b>25-49</b>		<b>n = 6110</b>	<b>n = 34,461</b>
	Pre	2.98 (0.04)	2.96 (0.01)
	Post	2.15 (0.04)	2.22 (0.01)
	D <sup>n</sup>	-0.83 (0.06)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.08 (0.05, -0.21), <i>p</i> = 0.2140	
<b>50</b>		<b>n = 1976</b>	<b>n = 34,461</b>
	Pre	3.61 (0.07)	2.96 (0.01)
	Post	2.17 (0.07)	2.22 (0.01)
	D <sup>n</sup>	-1.44 (0.10)	-0.75 (0.02)
	Diff-in-Diff (95% CI)	-0.69 (-0.50, -0.89), <i>p</i> < 0.0001	

Statistics reported as mean (standard error).

*AUDIT-C* Alcohol Use Disorders Identification Test-Consumption, *Pre* pre-index AUDIT-C score, *Post* post-index AUDIT-C score,  $D^1$  change in AUDIT-C score, *Diff-in-Diff* difference-in-difference, CI confidence interval.

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