Effects of moderate ethanol consumption as a function of n-6:n-3 dietary ratio on lipid profile, inflammation, and liver function in mice

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ABSTRACT

Objective: It is critical to understand how moderate ethanol exposure interacts with dietary components such as essential fatty acids to influence inflammatory processes underlying CVD pathogenesis. The purpose of this study was to examine the effects of moderate ethanol consumption and dietary n-6:n-3 fatty acid composition on markers associated with CVD in mice.

Methods: Twenty-three C57BL/6J mice consumed an 18% ethanol solution or 26.9% maltose dextrin solution (isocaloric control) for 12 weeks. Within each group, the mice were fed either a high n-6 (n-6:n-3 = 50:1) diet or a balanced n-3 (n-6:n-3 = 1:1) diet *ad libitum*. Following the exposure period, serum samples were analyzed to assess lipid profile, inflammatory markers, antioxidant capacity, DNA damage, and liver function enzyme activity.

Results: The control group gained more weight than the ethanol group ($P = 0.020$). In ethanol-exposed mice, HDL was significantly increased ($P = 0.009$). C-reactive protein (CRP; $P < 0.001$), high mobility group box 1 protein (HMGB1; $P = 0.011$), 8-oxo-deoxyguanosine (8-oxo-dG; $P = 0.019$), ALT ($P = 0.002$) and AP ($P = 0.021$) were lower in the ethanol group. There was a significant main effect of the n-3 diet on total antioxidant capacity (TAC; $P < 0.001$) and 8-oxo-dG ($P = 0.047$).

Conclusion: These findings indicate that moderate ethanol consumption and a balanced n-6:n-3 diet improve several inflammatory and lipid markers associated with CVD. Observed differences in weight gain between groups should be considered when interpreting these results.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for over 655,000 deaths and $219 billion in healthcare costs annually [1]. Epidemiological studies in humans and experimental data from rodent models indicate that there is a non-linear J-shaped relationship between alcohol consumption and CVD risk, with low-to-moderate levels of alcohol exposure conferring cardioprotective effects, including lower risk of mortality following acute myocardial infarction, while excessive alcohol consumption conversely increases the risk of cardiovascular disease and adverse coronary events [2–7]. Possible mechanisms by which low-to-moderate ethanol intake may reduce CVD risk include improving lipid profile, reducing body fat, modulating gene expression involved in cholesterol synthesis, reducing platelet aggregation, and having anti-inflammatory effects [4,8–14].

Despite well-established dose-dependent associations between alcohol consumption and cardiovascular risk, the interaction between moderate ethanol consumption and other dietary factors in regulating physiological processes underlying CVD risk is largely unexplored in controlled preclinical models. In particular, the combined interaction between moderate levels of ethanol exposure and the ratio of dietary omega-6 (n-6) to omega-3 (n-3) fatty acids is an important understudied area of research because n-3 fatty acids appear to provide cardioprotective benefits that overlap with those of moderate ethanol consumption [15,16]. Experimental studies indicate that supplementation of n-3 fatty acids may lower cardiovascular risk by improving lipid profile, blood pressure, endothelial function, and inflammatory markers [17]. Epidemiological studies reveal similar effects of dietary consumption of n-3 fatty acids, reporting lower total cardiovascular mortality and reduction of secondary cardiovascular outcomes [15]. By

contrast, increases in n-6 to n-3 ratios facilitate a proinflammatory state in the body and are associated with progression of coronary atherosclerosis [18,19]. Metabolically, an increased n-6:n-3 ratio may lead to lipogenesis and hepatic steatosis, thereby increasing CVD mortality [19]. In addition, high levels of n-6 fatty acids are associated with unfavorable changes in lipid profile, including increased total cholesterol, LDL, and triglycerides [20]. The ratio of n-6:n-3 dietary fatty acids in Western diets has markedly increased over the last century, which has been associated with increased inflammation and risk of many chronic diseases [21]. Dietary lipids are known to play a role in modulating disease course related to excessive alcohol consumption (e.g., alcoholic liver disease) [22]. However, little to no controlled experimental data has addressed how dietary fatty acid composition may serve to differentially modulate the effects of moderate alcohol intake on cardiovascular risk.

The objective of this study was to examine the effects of moderate ethanol consumption and dietary n-6:n-3 fatty acid composition on markers associated with cardiovascular disease and liver dysfunction in mice. It was hypothesized that moderate ethanol consumption in combination with a balanced (1:1) dietary ratio of n-6:n-3 fatty acids may result in potential additive protective effects on lipid and inflammatory markers associated with CVD, whereas a high (50:1) n-6:n-3 diet may by contrast antagonize any beneficial effects of moderate ethanol exposure on physiological indicators of CVD risk.

2. Methods

2.1. Animals

Twenty-three adult C57BL/6J mice (12 male, 11 female; The Jackson Laboratory, Bar Harbor, ME) were used. Mice were 20 weeks of age at the start of the experiment (i.e., ~20–30 years human equivalent). Throughout the experiment, mice were housed individually in standard plastic bottom cages for precise measurement of fluid and food intake, within a testing room maintained on a 12:12 h light/dark cycle and at an ambient temperature of approximately 23 °C. Fluids and chow diets were available to mice *ad libitum*. Mice were randomly assigned to one of four experimental groups (detailed in section 2.2), with each group balanced as equally as possible by gender and initial body weight. All study procedures were approved by the Institutional Animal Care and Use Committee (Animal protocol #:18-12-015B) at San Diego State University and were in accordance with National Institutes of Health guidelines.

2.2. Ethanol and diet exposure

All animals were initially acclimated to housing and testing procedures for 14 days during which they received free access to standard chow (LabDiet, St. Louis, MO) and water, and body weights were measured every 48 h. Following the initial acclimation period, mice were exposed for 15 weeks to either ethanol or isocaloric maltose dextrin (control) in the drinking water and were concurrently fed a compositional defined diet (LabDiet, St. Louis, MO) enriched with either corn oil or fish oil, comprising a high (50:1) or balanced (1:1) n-6:n-3 ratio, respectively [Fluid (2; ethanol or control) x Diet (2; high or balanced n-6:n-3) factorial design]. Chow diets were matched for all other macro- and micronutrients (Supplement Table 1). Ethanol-exposed mice were given graduating ethanol concentrations of 2.5%, 5%, and 10% during the first three weeks of exposure to acclimate to drinking ethanol and were maintained on an 18% ethanol solution during the remaining 12 weeks of exposure. The control group received isocalorically matched maltose dextrin mixed in their drinking water during corresponding weeks at concentrations of 3.7%, 7.5%, and 14.9% during the first three weeks and a maintenance level of 26.9% for the remaining 12 weeks. This model of moderate ethanol consumption in C57BL/6J mice has been demonstrated in previous studies to produce

mean blood ethanol concentrations (BECs) of 24–67 mg/dl [23–25]. The latter blood alcohol levels approximate 0.02–0.06% in humans, or that normally resulting from 1 to 2 standard drinks [26], corresponding with the current definition of moderate or 'low-risk' drinking as defined by the National Institute on Alcohol Abuse and Alcoholism and U.S. Department of Health and Human Services Dietary Guidelines for Americans 2015–2020 [27]. Body weights and bottle weights (to assess fluid intake) were measured every 48 h and food intake (g) was measured once per week.

2.3. Blood glucose and lipids

At the end of the exposure period, blood samples were collected via cardiac puncture under terminal isoflurane anesthesia. After centrifugation for 10 min at $1200 \times g$ at 4 °C, serum was collected and stored at −80 °C until analysis. Blood glucose was measured using the Glucose LiquiColor assay kit (EKF Diagnostics, Cardiff, UK). Serum triglycerides, total cholesterol, and high-density lipoprotein (HDL) were determined using assay kits (EKF Diagnostics). Non-HDL cholesterol was calculated using the formula: Non-HDL Cholesterol = TC – HDL – (TG/5).

2.4. C-reactive protein

C-reactive protein (CRP) levels were measured using a double antibody sandwich ELISA kit (Crystal Chem, Elk Grove Village, IL). Serum samples were incubated with anti-CRP antibodies and then with HRP-conjugated anti-CRP antibodies. TMB substrate was added to measure CRP concentration. Absorbance was read by spectrophotometer at 450 nm.

2.5. HMGB1

High-mobility group box1 protein (HMGB1) was measured by competitive ELISA (MyBioSource, San Diego, CA). Serum samples were incubated with HMGB1-HRP conjugate. After washing, samples were incubated with an HRP enzyme substrate, forming a blue-colored complex. Stop solution was added and absorbance was read at 450 nm.

2.6. Total antioxidant capacity

Serum total antioxidant capacity (TAC) was measured using an antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI). The capacity to which the antioxidants in the serum sample prevent oxidation of 2,2′-azino-di-[3-ethylbenthiazoline sulphonate] (ABTS) was measured. Trolox was used as a standard and absorbance was read at 405 nm.

2.7. 8-Oxo-deoxyguanosine

Levels of 8-oxo-deoxyguanosine (8-oxo-dG) were measured to assess DNA damage using HT 8-oxo-dG ELISA kit II (Trevigen, Gaithersburg, MD). Following the manufacturer's protocol, serum samples were incubated with monoclonal and then anti-Mouse IgG-HRP conjugate. TACS-Sapphire substrate was added and incubated in the dark. Stop solution was added, and absorbance was read at 450 nm.

2.8. Liver function enzymes

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Liqui-UV assay kits (EKF Diagnostics) and alkaline phosphatase (AP) was measured using the LiquiColor kit (EKF Diagnostics) according to the manufacturer's protocols. For AST and ALT assays, absorbance was read by spectrophotometer at 340 nm and absorbance per minute was calculated for AST and ALT. To determine AP enzyme activity, the rate of hydrolysis of 4-nitrophenyl phosphate to 4-nitrophenol was measured and the absorbance was read at

405 nm.

2.9. Statistical analysis

Data were analyzed using SPSS Version 26.0 (IBM, Armonk, New York). The primary analyses were 2 (Fluid: ethanol or control) x 2 (Diet: 50:1 or 1:1 n-6:n-3) factorial ANOVAs. If there was an interaction, Bonferroni post-hoc tests were used for multiple comparisons. ANOVAs were run for the following dependent variables: body weight, body weight gain, fluid intake, food intake, glucose, triglycerides, total cholesterol, HDL, LDL, ALT, AST, AP, AST:ALT ratio, CRP, HMGB1, TAC,

and 8-oxo-dG. For all main effects or interactions from the overall analyses, a p-value of <0.05 was considered statistically significant. Spearman's correlations were conducted to analyze relationships between the intake of total PUFAs, n-3 PUFAs, n-6 PUFAs and alcohol, with metabolic parameters.

3. Results

3.1. Body weight gain, food intake, and fluid intake

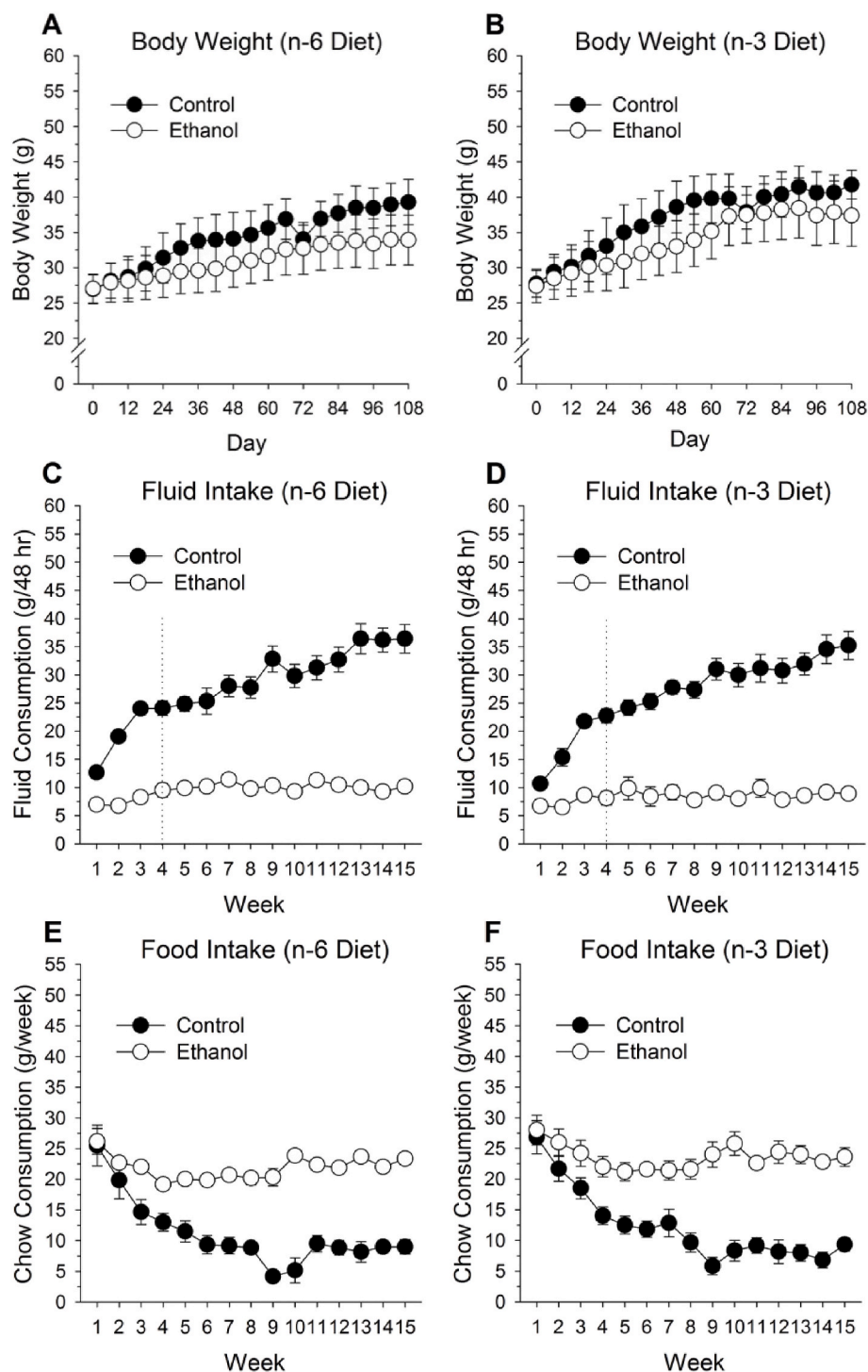There were no significant differences in either initial body weight or

Fig. 1. Mean (±SEM) body weight, fluid intake, and food intake over time in control and ethanol-exposed mice consuming the high n-6 or balanced n-3 diet. (A, B) Two-way Fluid (2) x Diet (2) factorial ANOVAs revealed no significant differences in either initial body weight or final body weight between groups. However, analysis of weight gain indicated that regardless of diet, control mice gained more weight than ethanol-treated mice over the course of the experiment (main effect of Fluid: $F_{1,19} = 6.03$, P = 0.02). Day 0 represents initial pretreatment body weight and Day 108 final body weight at the end of the treatment period. (C,D) Fluid intake was greater in control mice than ethanol-treated mice during all weeks except Week 1 (main effect of Fluid: $F_{1,19} = 222.34$, P < 0.001; main effect of Week: $F_{14,266} = 59.98$, P < 0.001; Fluid x Week interaction: $F_{14, 266} = 37.52$, P < 0.001). Mice were given ascending concentrations of either ethanol or maltose dextrin during the first 3 weeks of exposure and were then maintained on 18% ethanol or 26.9% maltose dextrin during the remaining 12 weeks (dotted line denotes start of maintenance phase). (E,F) Control mice consumed less food than ethanol-exposed mice during Weeks 3–15 (main effect of Fluid: $F_{1,19} = 121.86$, P < 0.001; main effect of Week: $F_{14,266} = 29.21$, P < 0.001; Fluid x Week interaction: $F_{14, 266} = 16.16$, P < 0.001). Fluid and food intake did not significantly differ by diet.

final body weight between groups (Fig. 1 and Supplemental Table 2). However, analysis of weight gain indicated that regardless of diet, control mice gained significantly more weight than those in the ethanol group (main effect of Fluid: $F_{1,19} = 6.03$, P = 0.020). Control mice also consumed significantly more fluid and less food compared to ethanol-exposed mice (main effect of Fluid: $F_{1,19} = 222.34$, P < 0.001; main effect of Fluid: $F_{1,19} = 121.86$, P < 0.001, respectively). Body weight, fluid intake, and food intake were not significantly influenced by diet. Energy intake was lower in the ethanol group than the control group (Supplemental Table 2). Total PUFA, n-6 and n-3 fatty acid intakes were greater in ethanol groups (main effect of Fluid: $F_{1,19} = 193.32$, P < 0.001; main effect of Fluid: $F_{1,19} = 104.32$, P < 0.001; main effect of Fluid: $F_{1,19} = 212.35$, P < 0.001, respectively) due to their greater food intakes.

3.2. Blood glucose and lipid profile

Glucose levels were significantly lower in the control group compared to the ethanol group, regardless of diet (main effect of Fluid: $F_{1,19} = 6.69$, P = 0.018; Table 1). Compared to controls, there was a trend toward lower triglycerides in ethanol-exposed mice (P = 0.066). Total cholesterol did not differ between the control group and the ethanol group overall; however, there was a trend toward lower LDL (P = 0.062) in mice fed the balanced n-3 vs high n-6 diet. HDL cholesterol was significantly higher among ethanol-exposed mice compared to control mice (main effect of Fluid: $F_{1,19} = 8.52$, P = 0.009), but was not affected by diet composition.

3.3. CRP, HMGB1, TAC, and 8-oxo-dG

C-reactive protein was significantly lower in ethanol-consuming mice compared to control mice (main effect of Fluid: $F_{1,19} = 25.16$, P < 0.001; Fig. 2A). HMGB1 was also significantly lower in the ethanol group compared to the control group (main effect of Fluid: $F_{1,19} = 7.95$, P = 0.011; Fig. 2B). The balanced n-3 diet increased TAC relative to the high n-6 diet in both control and ethanol-treated mice (main effect of Diet: $F_{1,19} = 32.88$, P < 0.001), with no significant effect of fluid type on TAC (Fig. 2C). The oxidative marker 8-oxo-dG was significantly lower in ethanol-exposed mice compared to controls (main effect of Fluid: $F_{1,19} = 6.61$, P = 0.019) and in mice consuming the n-3 diet compared to the n-6 diet (main effect of Diet: $F_{1,19} = 4.50$, P = 0.047; Fig. 2D). Ethanol in combination with the n-3 diet showed the lowest 8-oxo-dG levels among all groups, suggesting beneficial effects of these dietary factors.

Table 1
Glucose, triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein.

	Control		Ethanol	
	n-6	n-3	n-6	n-3
Glucose (mmol/L)	14.22 ± 2.09[a]	13.68 ± 1.37[a]	18.12 ± 0.59[b]	17.08 ± 0.94[b]
TG (mmol/L)	1.54 ± 0.16	1.63 ± 0.22	1.41 ± 0.18	1.02 ± 0.19
TC (mmol/L)	2.81 ± 0.45	2.80 ± 0.35	3.24 ± 0.46	2.43 ± 0.35
HDL (mmol/L)	0.95 ± 0.13[a]	1.16 ± 0.09[a]	1.55 ± 0.23[b]	1.57 ± 0.20[b]
LDL (mmol/L)	1.44 ± 0.22	1.19 ± 0.10	1.33 ± 0.23	0.84 ± 0.12

Data are represented as mean ± standard error. TG, triglyceride; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein. Two-way Fluid (2) x Diet (2) factorial ANOVAs were used for analysis. Data within rows with varying superscript letters are statistically significant (P ≤ 0.05). There was a significant main effect of fluid on glucose level (P = 0.018; NS effect of diet, P = 0.583; NS interaction: P = 0.864) and HDL cholesterol (P = 0.009; NS effect of diet, P = 0.494; NS interaction: P = 0.585). There was a trend toward lower TG in the ethanol group compared to controls (P = 0.066) and a trend toward lower LDL within the ethanol group in mice consuming the n-3 vs n-6 diet (P = 0.062).

3.4. AST, ALP, and AP

There was an interaction of fluid and diet on AST levels (main effect of Fluid: $F_{1,19} = 9.40$, P = 0.006; fluid x diet interaction: $F_{1,19} = 6.91$, P = 0.017). AST levels were lower in ethanol-exposed mice fed either diet and in controls fed the balanced n-3 diet (Fig. 3A). Regardless of diet, both ALT and AP were significantly lower in ethanol-consuming mice compared to controls (main effect of Fluid: $F_{1,19} = 13.60$, P = 0.002; main effect of Fluid: $F_{1,19} = 6.34$, P = 0.021, respectively; Fig. 3B and C). AST:ALT ratio was significantly greater in ethanol-exposed mice relative to controls (main effect of Fluid: $F_{1,19} = 5.10$, P = 0.036; Fig. 3D).

There was an inverse correlation (rho = −0.703, P = 0.011) between n-3 and AST in the control groups. Within ethanol groups, there were a trend or significant positive correlation between ethanol intake and AST (rho = 0.583, P = 0.060) and AP (rho = 0.656, P = 0.028).

4. Discussion

The present study was conducted to examine the effects of moderate ethanol consumption in conjunction with varied dietary n-6:n-3 fatty acid composition on risk factors for cardiovascular disease. It was found that moderate levels of ethanol consumption decreased key inflammatory markers CRP and HMGB1, reduced oxidative DNA damage as assessed via 8-oxo-dG, increased HDL cholesterol, and lowered both ALT and AP liver function enzyme activity. The balanced n-3 diet independently increased TAC and decreased 8-oxo-dG relative to a high n-6 diet. In combination, concurrent intake of moderate levels of ethanol together with the n-3 diet resulted in further decreases in 8-oxo-dG, as well as trends toward lower levels of triglycerides and LDL.

Previous data have documented that excessive ethanol consumption and heavy "binge" drinking episodes are associated with increased oxidative stress and inflammation [8,28] leading to detrimental effects on many organ systems and increased risk for adverse coronary events [29,30]. By contrast, several lines of evidence indicate that low-to-moderate ethanol exposure is associated with reduced incidence of cardiovascular disease [2,3] and may provide cardioprotective effects through modulation of inflammatory markers and gene expression and a reduction in oxidative damage [8,12]. Controlled preclinical studies in rats have demonstrated that moderate ethanol intake results in decreased oxidative stress by upregulation in the expression of *Pon-1* and *Aldh2* and lowered inflammation by decreased expression in *Cox-2* and *Rela* [12,31]. Human observational studies have demonstrated that moderate alcohol consumption, and in particular red wine, which contains higher amounts of polyphenols compared to other types of alcohol, may prevent the activation of the proinflammatory NF-κB pathway as well as decrease CRP and increase TAC [32–34]. It is possible that moderate levels of ethanol may reduce inflammation and oxidative damage by acting as a hormetic stressor. Ethanol generates reactive oxygen species and aldehydes in the body, which can cause oxidative injury; however, in acute conditions, low levels of reactive oxygen species may induce cytoprotective effects [35]. As such, low-to-moderate levels of alcohol may engage cellular defense mechanisms and stimulate compensatory protective effects [12]. A previous study found that moderate ethanol consumption also reduced DNA damage and inflammation in markers associated with colon cancer risk [31]. In the present study, mice exposed to moderate ethanol had significantly lower CRP, HMGB1, and DNA damage as measured by 8-oxo-dG, but no significant differences in TAC. It is important to note that tissues in the current study were collected one day after the final alcohol exposure session at a time when ethanol was absent from blood in order to assess the effects of long-term low-to-moderate ethanol exposure as opposed to direct effects of ethanol in the system. It would be interesting for subsequent studies to determine the long-term persistence of these protective effects during a period of sustained abstinence.

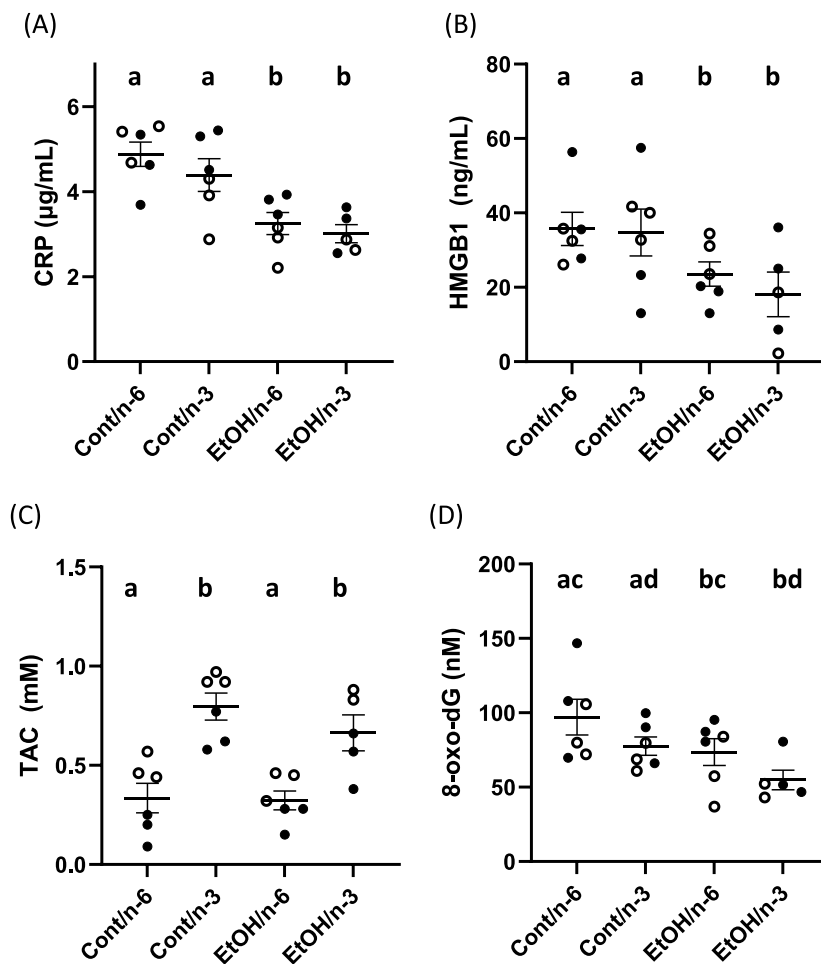Consumption of ethanol increases HDL cholesterol in a dose-

Fig. 2. Mean values of C-reactive protein, HMGB1, and total antioxidant capacity. (A) There was a significant main effect of ethanol treatment on CRP (main effect of fluid, P < 0.001; NS effect of diet, P = 0.239; NS interaction, P = 0.678). (B) HMGB1 was significantly lower in ethanol-exposed animals compared to controls (main effect of fluid, P = 0.011; NS effect of diet, P = 0.537; NS interaction, P = 0.666). (C) The n-3 diet resulted in significantly higher TAC compared to the n-6 diet (main effect of diet, P < 0.001; NS effect of fluid, P = 0.310; NS interaction, P = 0.407). (D) There was a significant main effect of ethanol on 8-oxo-dG (main effect of fluid, P = 0.019) and of n-3 diet on 8-oxo-dG (main effect of diet, P = 0.047). Two-way Fluid (2) x Diet (2) factorial ANOVAs were used for primary analysis. Since there were no significant interactions, no-post-hoc tests were performed. Lines for each group represent mean ± standard error. Varying superscripts are significantly different (P ≤ 0.05). Each group was comprised of 3 male and 3 female mice, except the Ethanol n-3 group which contained 3 male and 2 female mice. Closed circles (•) indicate male mice and open circles (o) female mice. 8-oxo-dG: 8-oxo-deoxyguanosine; CRP, c-reactive protein; HMGB1: high mobility group box 1; NS: not significant; TAC: total antioxidant capacity.

dependent manner [8]. The results of the current study were consistent with this finding, demonstrating significantly higher HDL levels in ethanol-exposed mice, which was evident regardless of n-6:n-3 diet composition. We also observed a borderline trend for lower triglycerides in the ethanol group, which was most pronounced in ethanol-exposed mice also consuming the balanced n-3 diet. Alcohol in high doses is known to increase plasma triglycerides; however, the effects of moderate ethanol on triglycerides has been inconclusive and may depend on the type of alcohol consumed [28,32,36].

The liver is the primary site of ethanol metabolism, and as such, liver function becomes impaired with heavy drinking [37]. Chronic excessive consumption of alcohol can lead to alcoholic liver disease (ALD) and cirrhosis [37]. Interestingly, prior rodent studies employing paradigms that result in low-to-moderate levels of ethanol drinking have shown improved liver function as indexed by reductions in both ALT and LDH (lactate dehydrogenase) [31,38]. Potential mechanisms for this improvement in liver function may be related to a reduction in serum ammonia [38]. The present study found reductions in both ALT and AP resulting from moderate alcohol consumption; however, an increase in AST:ALT ratio was also observed. An AST:ALT ratio >1 is suggestive of alcoholic liver disease, observed in 92% of patients with ALD [39]. In this study, control mice displayed AST:ALT ratios <1, while both ethanol groups had ratios >1. These findings indicate that there may be adverse consequences of moderate ethanol intake on the liver, despite observed overall reductions in ALT and AP. Additional research should be conducted in this area to further elucidate these paradoxical

consequences of moderate alcohol consumption on liver function. Conflicting data also exist with regard to the effects of moderate alcohol exposure on the development and progression of nonalcoholic liver disease, with several reports indicating reduced incidence of fatty liver development and liver fibrosis, while other data report increased progression of fibrosis and hepatocellular carcinoma [40–42]. These diverse findings may be influenced by multiple factors including age, metabolic status, existing pathology, and methodological issues such as inaccuracies in self-reported alcohol intake and heterogeneity in defining moderate consumption levels [40–43].

Heavy ethanol consumption and binge drinking are associated with an increase in blood glucose and consequently an increased risk of type 2 diabetes and metabolic syndrome [44,45]. High doses of ethanol may result in an increase in glucose levels by inducing insulin resistance [44, 45]. It has been previously shown that binge drinking impairs insulin signaling in the hypothalamus, inducing systemic insulin resistance [45]. The current study found increased plasma glucose in ethanol-exposed mice, although prior studies have indicated possible benefits in glucose metabolism and lowered blood glucose from moderate alcohol consumption [12,46]. Proposed mechanisms by which moderate ethanol exposure may decrease fasting glucose are through increased insulin secretion or the inhibition of gluconeogenesis via alcohol metabolism [47]. Studies directly measuring effects of ethanol on insulin sensitivity have produced conflicting results, showing slight benefits or no difference [46]. There are several potential reasons for the observed variation in blood glucose among moderate alcohol
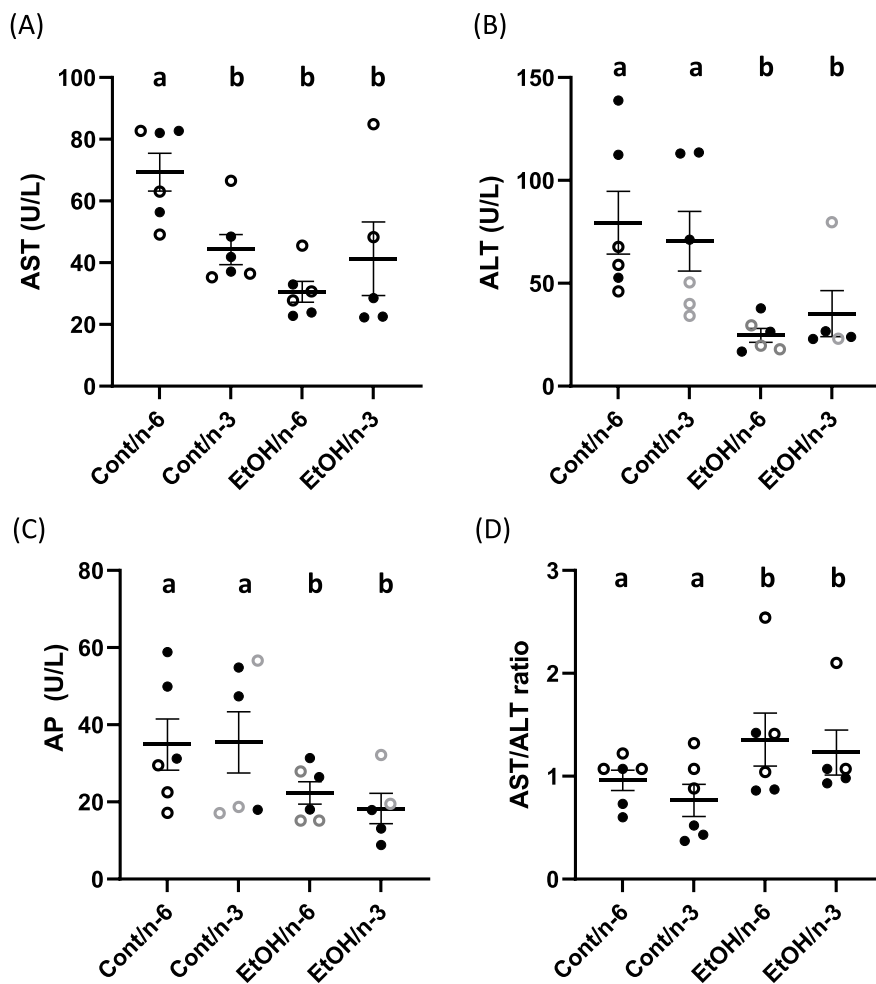
Fig. 3. Mean values of liver enzymes AST, ALT, and AP (U/L). (A) There was an interaction of fluid and diet on AST levels (main effect of fluid, P = 0.006; fluid x diet interaction: P = 0.017; NS effect of diet, P = 0.304). AST was significantly lower in ethanol-exposed mice fed either diet and in controls fed the n-3 diet only. Regardless of diet, ALT (B) and AP (C) were significantly lower in ethanol-consuming mice compared to controls (main effects of fluid: P = 0.002 and P = 0.021, respectively). (D) AST/ALT ratio was significantly greater in ethanol-exposed mice compared to control mice (main effect of fluid, P = 0.036; NS effect of diet, P = 0.408; NS interaction: P = 0.868). Two-way Fluid (2) x Diet (2) factorial ANOVAs were used for analysis. If there was an interaction, Bonferroni post-hoc tests were used for multiple comparisons. Lines for each group represent mean ± standard error. Varying superscripts are significantly different (P ≤ 0.05). Each group was comprised of 3 male and 3 female mice, except the Ethanol n-3 group which contained 3 male and 2 female mice. Closed circles (•) indicate male mice and open circles (o) female mice. AST, aspartate aminotransferase; ALT, alanine transaminase; AP, alkaline phosphatase; NS: not significant.

consumers, including the type of alcohol consumed, whether it was consumed concurrently with food, and if glucose measurements were taken in a fasted state or not [48]. There were no significant differences in blood glucose in the present study as a function of n-6:n-3 dietary ratio.

Previous studies have shown that n-3 fatty acids can lower triglycerides; however, current research does not strongly support beneficial effects of n-3 fatty acids on total cholesterol, LDL, or HDL [15,49]. While the present study did not find any main effects of n-3 diet alone on lipid profile, several trends were observed for the balanced n-3 diet to reduce triglycerides, total cholesterol, and LDL in mice also consuming ethanol. It is well known that n-3 fatty acids promote anti-inflammatory effects in the body, likely by reducing the production of pro-inflammatory cytokines as well as attenuating oxidative stress and improving endothelial function [15]. Additionally, when n-3 fatty acids bind with peroxisome proliferated-activated receptors (PPAR) transcription factors, they are activated for cytoprotective responses [50]. Recent research indicates that n-3 fatty acids may decrease inflammation by modulating the HMGB1/NF-κB pathway [51]. In addition, several studies have reported an increase in total antioxidant capacity and decreased DNA damage from n-3 fatty acids [52–55]. One mechanism by which n-3 fatty acids may improve antioxidant status is through upregulation of the transcription factor NRF2, a key regulator of antioxidant gene expression [53,54]. The results of this study are in line with these findings, showing a significant reduction in 8-oxo-dG, an increase in total antioxidant capacity, as well as a trend toward lower CRP in the

balanced n-3 diet group. Concurrent intake of ethanol together with the n-3 diet resulted in further reductions in 8-oxo-dG, potentially suggesting beneficial effects in combination. It should be noted that while ethanol combined with the n-3 diet resulted in apparent facilitative effects on multiple measures, the main effects of ethanol in this study were strong, which may have overshadowed more robust interactions of these dietary factors in combination.

Finally, the results of the present study should be interpreted within the context of observed differences in fluid and food intake between ethanol-exposed and control mice which resulted in a significantly greater body weight gain in the control group compared to the ethanol group. Although control mice were provided with a standard maltose dextrin control solution that was isocalorically matched to ethanol, greater preference for the maltose dextrin solution led to increased fluid consumption and higher body weight gain compared to ethanol-consuming animals. An alternate feeding model employing pair feeding of fluids according to the rate-limiting group and/or an alternative control fluid may be useful in future studies in order to better equate levels of fluid consumption and body weight gain between groups. Further studies are also needed to better understand the effects of moderate alcohol consumption on liver function including histology and additional parameters of oxidative stress, as well as blood glucose and insulin sensitivity including a glucose tolerance test with assessment of glucose stimulated insulin secretion, given across-study variation in these measures. Additionally, future studies that systematically evaluate alcohol dose-related effects, including an experimental group

administered a higher dose of alcohol, will allow for within-study comparison of the effects of moderate vs. excessive alcohol exposure on key measures of interest.

Alcohol consumption can affect the gastrointestinal tract by direct exposure to ethanol and/or its metabolite acetaldehyde. Alcohol abuse can damage the mucous cells in the gastrointestinal tract, induce inflammation and lesions and/or alter gastric acid output [56]. Many studies have examined effects of excessive or heavy ethanol drinking on the gastrointestinal tract, however, effects of moderate ethanol consumption have been less well studied. Moderate ethanol consumption has been shown to increase glutathione-S-transferase M1 expression and aldehyde dehydrogenase 2 expression in the colon, which may reduce mucosal colonic damage [31]. More research is necessary to investigate the effect of moderate ethanol consumption on the gastrointestinal tract.

5. Conclusion

Taken together, the present findings indicate independent beneficial effects of moderate alcohol consumption and a balanced n-6:n-3 diet on certain inflammatory and lipid profile markers associated with cardiovascular disease in mice. These findings add to a growing body of literature exploring the protective effects of moderate ethanol consumption on CVD risk and underlying modulatory mechanisms. Nevertheless, it is critical to recognize that dose-dependent effects of ethanol are complex, and beneficial effects of moderate ethanol are not ubiquitous across all disease processes, and further vary by gender, genetic background, and other inter-individual differences. The mechanisms by which moderate alcohol drinking promotes or protects against disease and how this is modulated by other interacting dietary factors is an important area for continued research.

CRediT authorship contribution statement

Kristine Giltvedt: Data curation, Investigation, Visualization, Writing – original draft. **Luciano S. Voutour:** Data curation, Investigation. **Brianna Tursellino:** Data curation. **Alexandra Zella:** Data curation. **Susan M. Brasser:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing, Funding acquisition. **Mee Young Hong:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijcrp.2022.200132.

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