



## OPEN Decreased antioxidant-related superoxide dismutase 1 expression in peripheral immune cells indicates early ethanol exposure

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Alcohol consumption increases oxidative stress and imbalances in the antioxidant system, even with ethanol (EtOH) exposure at a young age. This study assessed changes in the antioxidant system following young EtOH exposure in peripheral immunity and measured sensitive indicators of heavy alcohol consumption. We used peripheral blood mononuclear cells (PBMCs) from 197 male university students without smoking habits to examine changes in antioxidant-related gene expression in vitro and in PBMCs. In vitro, the antioxidant system was impaired by EtOH. Next, we examined the expression of 84 antioxidant-related genes in the PBMCs of 162 young adults, among which the superoxide dismutase (SOD) 1 expression was most negatively correlated with alcohol consumption degree. The plasma SOD1 level had the highest area under the curve value (0.806) for heavy alcohol consumption. Our data demonstrated that a decreased SOD1 level is a sensitive indicator of an impaired antioxidant system and heavy alcohol consumption with early EtOH exposure.

**Keywords** SOD1, Antioxidant gene expression, Peripheral immune cells, Young adults, Early ethanol exposure, Oxidative stress

Moderate alcohol consumption relieves mental and physical stress, whereas excessive drinking exerts adverse effects on immunity and organs, such as the liver and pancreas, by increasing oxidative stress (OS) in the body<sup>1–4</sup>. Several antioxidant enzymes suppress OS, and various changes in these enzymes following alcohol intake are observed in the cells, tissues, and organs<sup>5</sup>. Peripheral immunity is also directly influenced by alcohol/ethanol (EtOH), which adversely affects the antioxidant system<sup>6</sup>; however, this has not been investigated in detail. High alcohol consumption increases the risk of health damage, and globally, light-to-moderate alcohol consumption increases the risk of developing malignant tumors<sup>7–9</sup>. Notably, “heavy-drinking” individuals, consuming more than 100 g/week, have a higher mortality rate; furthermore, some young adults have inappropriate drinking habits, including heavy drinking<sup>10,11</sup>. The antioxidant system may also be adversely influenced by light-to-heavy alcohol consumption in young adults with early alcohol-drinking habits.

Previously, we reported a noninvasive diagnostic method for nonalcoholic steatohepatitis based on gene expression profiles in peripheral blood mononuclear cells (PBMCs), finding that these profiles are useful for predicting pathology<sup>12</sup>. EtOH exposure induces OS and imbalances in the antioxidant system, including downregulating the gene expression of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in PBMCs<sup>6,13</sup>; however, the changes in the antioxidant system in human peripheral immunity due to EtOH exposure at a young age have not been elucidated. Accordingly, identifying sensitive indicators of “heavy drinking” at a young age to prevent the risk of lifestyle-related diseases and high mortality will be necessary in the future<sup>10,14</sup>. Hematological and biochemical parameters related to alcohol consumption, such as liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT),

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and mean corpuscular volume (MCV), are clinically used to assess alcohol consumption and liver damage in adults<sup>15,16</sup>; however, their usefulness in young adults has not been elucidated.

In this study, we focused on young adults who were exposed to EtOH at a young age and examined the changes in antioxidant-related gene expression profiles of their PBMCs to assess the role of the antioxidant system in peripheral immunity under EtOH exposure. We examined several antioxidant-related gene expression levels in vitro and in the PBMC samples of young male adults and identified the indicators.

## Results

### Decreased antioxidant gene expression in PBMCs

To assess OS-related changes in peripheral immunity, we first examined the in vitro intercellular ROS, cell viability, and expression levels of five OS-related genes: *SOD1*, *SOD2*, *GPX1*, *CAT*, and *NOX2* in the PBMCs of healthy controls after treatment with several concentrations (0 [control], 6.2, 12.5, 25, 50, and 100 mM) of EtOH for different times (24 and 72 h exposure) (Fig. 1). The range of EtOH 6.25–100 mM corresponded to moderate-heavy drinking in young adults. Relative fold changes in intercellular ROS and *NOX2* mRNA expression levels were significantly increased for all EtOH treatments compared to the control after 24 h of exposure ( $p < 0.05$ , Fig. 1A,C) and for all EtOH treatments except 6.2 mM after 72 h of exposure ( $p < 0.05$ , Fig. 1E,G).

No significant differences were observed in cell viability between the control and EtOH treatments at 24 h (Fig. 1B); however, the relative fold change was significantly decreased with the EtOH treatments (50 and 100 mM) compared to the control after 72 h of exposure ( $p < 0.05$ , Fig. 1F).

Except for *NOX2*, mRNA expression levels of the other four antioxidant-related genes were significantly decreased with all EtOH treatments compared to the control after 24 h of exposure ( $p < 0.05$ , Fig. 1D). *GPX1* and *CAT* mRNA expression levels were significantly decreased with all EtOH treatments compared to the control after 72 h of exposure, and those of *SOD1* and *SOD2* were significantly decreased with all EtOH treatments except with 6.2 mM ( $p < 0.05$ , Fig. 1H). These results suggest that the antioxidant enzymes in PBMCs tend to decrease with increasing and early exposure to EtOH in vitro.

### Students' characteristics

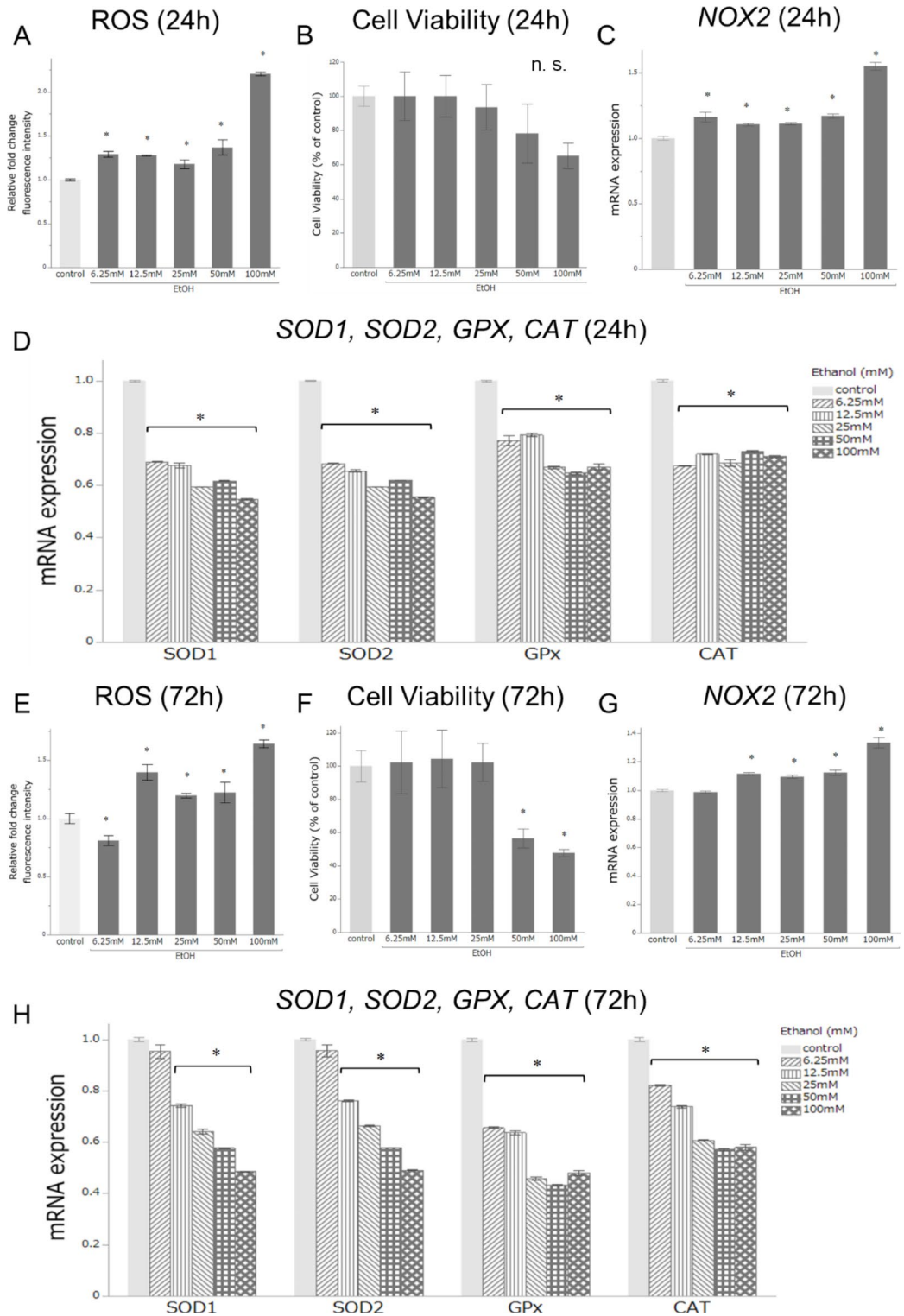
The baseline characteristics of the 197 male students without smoking habits (35 adolescents aged 18–19 years and 162 young adults aged 22–29 years) are shown in Table 1. All 35 adolescents were non-drinkers and 162 young adults were drinkers. The drinkers were divided into three groups (light,  $< 0.31$  g/kg/week:  $n = 63$ ; moderate,  $0.31$ – $1.59$  g/kg/week:  $n = 66$ ; heavy,  $> 1.59$  g/kg/week:  $n = 33$ ) according to their average weekly alcohol consumption. In the 162 drinkers, no significant differences in age and BMI were observed among the three groups. No significant differences in AST and ALT were observed among the three groups, whereas the GGT level was significantly increased in the heavy-drinking group compared to the light-drinking group ( $p < 0.05$ ). Further, the MCV level was significantly increased in the heavy-drinking groups compared to the light-drinking group ( $p < 0.05$ ). In this study, clinical differences among non-drinkers were assessed between adolescent non-drinkers and young adults who are light drinkers, who were regarded as “non-drinking” young adults. No significant differences in AST, ALT, GGT, and MCV levels were observed between adolescent non-drinkers and light drinkers; however, significant differences were observed in DBP, TG, LDL-C, platelet count, and FBS levels ( $p < 0.05$ ). These parameters may be influenced by aging and lifestyle habits. Therefore, in this study, the expression levels of several OS-related genes under early EtOH exposure were assessed in 162 young adults, excluding adolescents.

### Screening expression levels of 84 antioxidant-related genes

For screening, we first examined the mRNA expression levels of 84 antioxidant-related genes in the PBMCs of 24 randomly selected drinkers (light:  $n = 8$ ; moderate:  $n = 8$ ; heavy:  $n = 8$ ; Table S1) using a commercially available expression array (Table S2). Genes whose mRNA expression levels were  $> 1.5$ -fold higher between at least two of the three groups were regarded as upregulated<sup>12</sup>. The mRNA expression levels of *BNIP3*, *CAT*, *CCL5*, cytochrome b-245 alpha polypeptide (*CYBA*), diacylglycerol kinase kappa (*DGKK*), dual specificity phosphatase (*DUSP*) 1, Soluble epoxide hydrolase (*EPHX*) 2, *GPX1*, *GPX7*, *MT3*, neutrophil cytosol factor (*NCF*) 1, *PDLIM1*, peroxiredoxin (*PRDX*) 6, *SOD1*, and *TTN* were  $> 1.5$ -fold higher between at least two of the three groups (Table 2). However, none of the differences were significant, likely because of the small number of patients examined.

### Expression levels of 15 antioxidant-related genes in drinkers

We determined the mRNA expression levels of these 15 candidate genes in the PBMCs of all 197 students via real-time PCR using specific primers and probes, and the *NOX2* mRNA expression level was also examined as an assessment of the degree of OS in PBMCs. Notably, significant differences for antioxidant-related genes were observed between adolescent non-drinkers and light drinkers ( $p < 0.05$ , Fig. S1); therefore, the expression levels of these genes in drinkers were assessed among 162 young adults. Only *SOD1* expression was significantly downregulated in the heavier-drinking group compared to that in any two of the three drinking groups ( $p < 0.05$ , Fig. 2). The expression levels of *BNIP3*, *CCL5*, *EPHX2*, *GPX1*, *GPX7*, *MT3*, *NCF1*, *PDLIM1*, *PRDX6*, and *TTN* were significantly downregulated in the heavier-drinking group in at least two of the three groups ( $p < 0.05$ ). The expression of all 11 genes was negatively correlated with average weekly alcohol consumption ( $p < 0.05$ , Fig. 3), and *SOD1* expression was the most negatively correlated ( $p < 0.001$ ). The *NOX2* expression level was significantly upregulated in the moderate- and heavy-drinking groups compared to the light group ( $p < 0.05$ ); furthermore, it was positively correlated with average weekly alcohol consumption ( $p = 0.021$ ). Similar results were observed in the changes and correlations in the expression of these 15 genes and *NOX2* according to average daily alcohol consumption and frequency of alcohol consumption (Table S3). These results suggest that the expression of



**Fig. 1.** Assessment of oxidative stress-related changes in peripheral blood mononuclear cells (PBMCs) in vitro after ethanol (EtOH) treatment. PBMCs were examined after treatment with several concentrations (0 [control], 6.2, 12.5, 25, 50, and 100 mM) over (A–D) 24 and (E–H) 72 h of exposure. (A,E) Relative fold changes in intercellular ROS expression levels compared to control. (B,F) Relative fold changes in cell viability compared to control. (C,G) Relative fold changes in NOX2 mRNA expression level compared to control. (D,H) Relative fold changes in mRNA expression levels of SOD1, SOD2, GPX, and CAT compared to control. Data are expressed as the mean ± SEM. ( $n = 3$ ; \* $p < 0.05$  compared to control; n.s., not significant; Student’s  $t$ -test).

Parameter	Non-drinkers (age 18–19)	Drinkers (age 22–29)		
	None (n = 35)	Light (n = 63)	Moderate (n = 66)	Heavy (n = 33)
Age (years)	18.5 ± 0.1	23.8 ± 0.3**	23.9 ± 0.3**	24.2 ± 0.4**
BMI (kg/m <sup>2</sup> )	21.1 ± 0.5	20.9 ± 0.3	20.8 ± 0.3	21.4 ± 0.4
BW (kg)	61.7 ± 1.5	62.2 ± 1.0	62.3 ± 1.0	65.3 ± 1.5
SBP (mmHg)	118.7 ± 2.1	119.6 ± 1.5	124.1 ± 1.4*	123.7 ± 1.9
DBP (mmHg)	66.9 ± 1.3	73.1 ± 1.1**	75.2 ± 1.2**	73.2 ± 1.5*
Alcohol consumption (g/kg/week)		0.13 ± 0.01	0.87 ± 0.05##	2.97 ± 0.32###††
Daily average consumption (g/kg/day)		0.08 ± 0.01	0.28 ± 0.01##	0.58 ± 0.06###††
Frequency (days/week)		1.4 ± 0.1	3.4 ± 0.2##	5.2 ± 0.3###††
AST (U/L)	20.4 ± 0.7	19.7 ± 0.7	20.8 ± 0.9	20.0 ± 1.6
ALT (U/L)	18.7 ± 1.4	18.5 ± 1.4	20.4 ± 1.6	19.6 ± 3.5
GGT (U/L)	19.9 ± 1.2	22.5 ± 1.4	30.0 ± 2.9*	29.3 ± 3.3*#
TG (mg/dL)	45.5 ± 2.4	94.7 ± 5.2**	85.4 ± 6.4**#	107.0 ± 17.5**
HDL-C (mg/dL)	62.5 ± 1.8	59.6 ± 1.5	62.8 ± 1.5	64.6 ± 2.2
LDL-C (mg/dL)	96.3 ± 4.8	106.2 ± 3.4*	103.0 ± 3.5	103.6 ± 4.6
UA (mg/dL)	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	6.1 ± 0.2*†
Creatinine (mg/dL)	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.84 ± 0.02
CRP (mg/dL)	0.05 ± 0.03	0.03 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
Platelet count (10,000/μL)	28.6 ± 1.0	24.3 ± 0.6**	26.3 ± 0.6*	24.4 ± 0.7*
FBS (mg/dL)	79.7 ± 0.8	84.5 ± 1.7	82.5 ± 0.8*	93.5 ± 7.3**†
MCV (fL)	92.8 ± 0.5	93.0 ± 0.4	94.0 ± 0.4	95.0 ± 0.7*#

**Table 1.** Clinical background of 162 young adult drinkers (ages 22–29) and 35 adolescent non-drinkers (ages 18–19). \* $p < 0.05$  compared with non-drinkers \*\* $p < 0.001$  compared with non-drinkers. # $p < 0.05$  compared with light drinkers among drinkers ## $p < 0.001$  compared with light drinkers among drinkers. † $p < 0.05$  High drinkers compared with moderate drinkers †† $p < 0.001$  High drinkers compared with moderate drinkers. Values are presented as means ± SEM.

several antioxidant-related genes, especially SOD1, in peripheral immunity during young EtOH exposure tends to decrease with an increase in consumption.

### Plasma levels of six antioxidant-related proteins in drinkers

We measured the plasma levels of several antioxidant-related proteins using commercially available ELISA kits (Table S4). In this study, genes with correlation coefficients  $> 0.30$  were regarded as having stronger correlations with EtOH consumption. Proteins of the six applicable antioxidant-related genes, *BNIP3*, *CCL5*, *MT3*, *PDLIM1*, *SOD1*, and *TTN*, were selected to assess significant candidate indicators of EtOH exposure at a young age. Only the SOD1 plasma level was significantly downregulated in the heavier-drinking group between any two of the three drinking groups ( $p < 0.001$ , Fig. 4A). The plasma levels of BNIP3 and CCL5 were significantly downregulated in the heavier drinking group in at least two of the three groups ( $p < 0.05$ ). No significant difference in MT3, PDLIM1, and TTN plasma levels was observed among the three groups. Among the proteins of the six antioxidant-related genes, the plasma levels of BNIP3, SOD1, and TTN were negatively correlated with average weekly alcohol consumption ( $p < 0.002$ , Fig. 4B), and SOD1 plasma levels were most negatively correlated ( $p < 0.001$ ). Similar results were observed for the changes and correlations of plasma levels of these three proteins according to the average daily alcohol consumption and frequency of alcohol consumption (Table S5). These results suggest that a decreased plasma level of SOD1 in the peripheral blood is a powerful indicator of increased EtOH consumption.

### SOD1 as a sensitive indicator of heavy alcohol consumption

In this study, we assessed the changes and correlations of 16 clinical and biochemical parameters in drinkers according to their average weekly and daily alcohol consumption and the frequency of alcohol consumption (Table S6). GGT, HDL-C, and MCV levels were positively correlated with average weekly and daily alcohol consumption ( $p < 0.025$ ), and GGT and MCV levels were positively correlated with daily frequency ( $p < 0.012$ ). As shown in Fig. 5, the selection of sensitive indicators of heavy consumption under EtOH exposure at a young age was assessed among three antioxidant-related genes: *BNIP3*, *SOD1*, and *TTN* (Fig. 5A) and the three previously mentioned alcohol-related parameters: GGT, HDL-C, and MCV levels (Fig. 5B), based on the area under the curve (AUC). Of the six AUC values, the SOD1 level had the highest AUC value of 0.806. These results suggest that a decreased plasma level of SOD1 may be a sensitive indicator of heavy alcohol consumption in young adults when they have been exposed to EtOH at a young age.

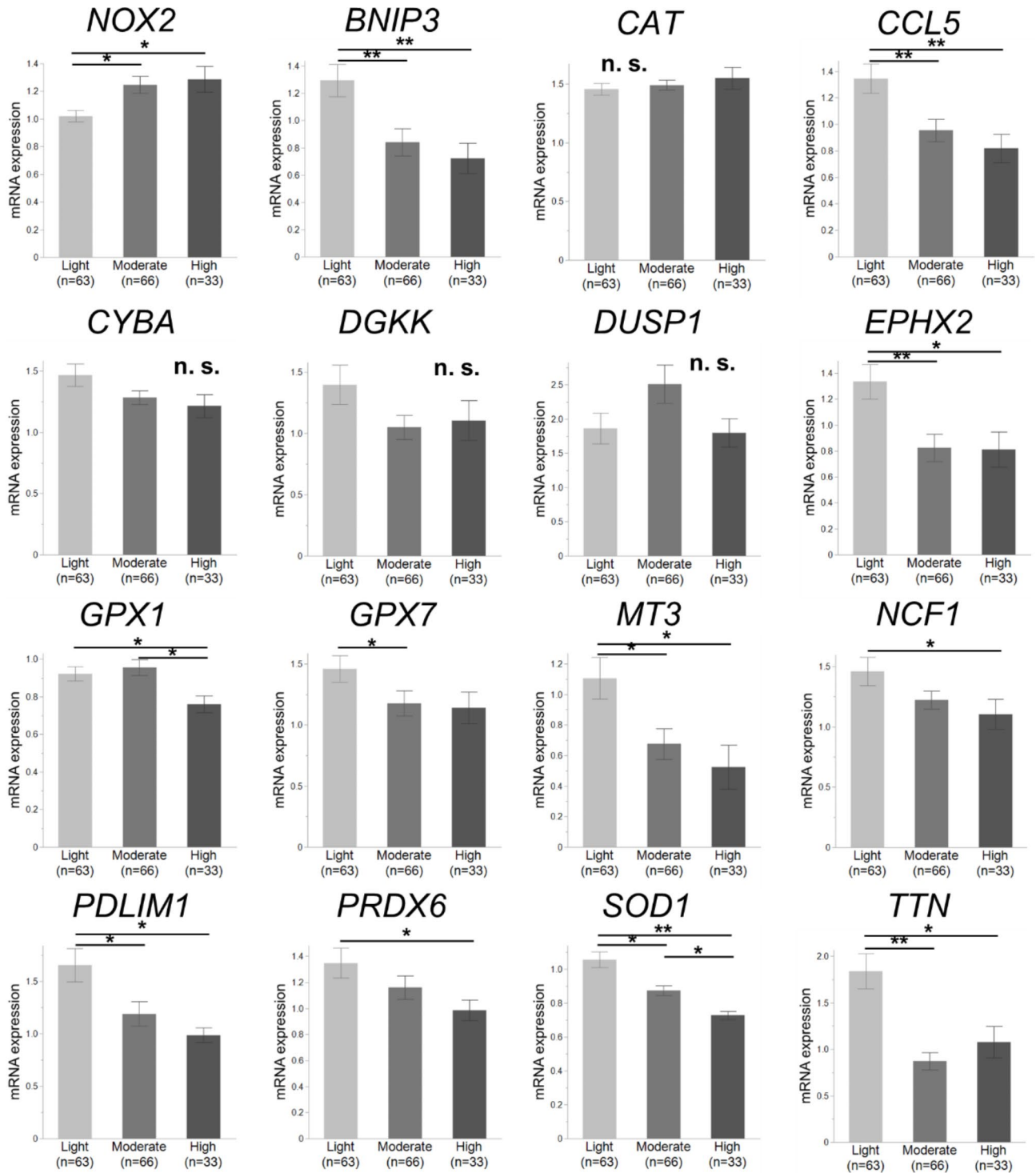
Gene	Drinkers			Gene	Drinkers		
	Light (n = 8)	Moderate (n = 8)	Heavy (n = 8)		Light (n = 8)	Moderate (n = 8)	Heavy (n = 8)
ALB	ND	ND	ND	MTL5	ND	ND	ND
ALOX12	1.13 ± 0.50	0.85 ± 0.47	0.99 ± 0.36	NCF1	<b>1.64 ± 0.42</b>	1.14 ± 0.49	<b>1.08 ± 0.49</b>
ANGPTL7	ND	ND	ND	NCF2	1.38 ± 0.59	1.40 ± 0.59	1.59 ± 0.61
AOX1	ND	ND	ND	NME5	ND	ND	ND
APOE	ND	ND	ND	NOS2	ND	ND	ND
ATOX1	5.56 ± 2.37	5.94 ± 2.39	6.42 ± 2.35	NOX5	ND	ND	ND
<b>BNIP3</b>	<b>1.31 ± 0.23</b>	<b>0.51 ± 0.23</b>	<b>0.38 ± 0.17</b>	NUDT1	0.38 ± 0.15	0.27 ± 0.12	0.35 ± 0.15
<b>CAT</b>	<b>1.52 ± 0.46</b>	<b>0.90 ± 0.27</b>	<b>1.75 ± 0.47</b>	OXR1	0.48 ± 0.15	0.33 ± 0.16	0.43 ± 0.18
<b>CCL5</b>	<b>1.27 ± 0.49</b>	<b>0.97 ± 0.32</b>	<b>0.59 ± 0.23</b>	OXSRI	0.58 ± 0.16	0.59 ± 0.25	0.72 ± 0.29
CCS	0.44 ± 0.14	0.39 ± 0.11	0.52 ± 0.15	<b>PDLIM1</b>	<b>1.56 ± 0.59</b>	<b>0.71 ± 0.23</b>	<b>0.74 ± 0.23</b>
CSDE1	2.92 ± 1.19	1.72 ± 0.62	2.86 ± 1.32	PIP3-E	0.54 ± 0.15	0.51 ± 0.25	0.48 ± 0.18
<b>CYBA</b>	<b>2.16 ± 0.91</b>	<b>0.73 ± 0.27</b>	<b>1.05 ± 0.27</b>	PNKP	0.42 ± 0.14	0.29 ± 0.12	0.36 ± 0.14
CYGB	ND	ND	ND	PRDX1	0.31 ± 0.12	0.25 ± 0.12	0.35 ± 0.14
<b>DGKK</b>	<b>1.38 ± 0.44</b>	<b>0.71 ± 0.25</b>	<b>1.04 ± 0.35</b>	PRDX2	0.61 ± 0.22	0.61 ± 0.25	0.61 ± 0.32
DHCR24	0.40 ± 0.14	0.43 ± 0.17	0.40 ± 0.14	PRDX3	0.30 ± 0.11	0.21 ± 0.11	0.22 ± 0.03
DUOX1	ND	ND	ND	PRDX4	0.48 ± 0.14	0.43 ± 0.21	0.50 ± 0.22
DUOX2	ND	ND	ND	PRDX5	0.50 ± 0.16	0.50 ± 0.25	0.42 ± 0.14
<b>DUSP1</b>	<b>1.40 ± 0.62</b>	1.92 ± 0.96	<b>1.75 ± 0.90</b>	<b>PRDX6</b>	<b>1.13 ± 0.46</b>	<b>0.66 ± 0.24</b>	<b>0.65 ± 0.20</b>
<b>EPHX2</b>	<b>1.40 ± 0.44</b>	<b>0.55 ± 0.17</b>	<b>0.75 ± 0.29</b>	PREX1	0.32 ± 0.12	0.25 ± 0.12	0.27 ± 0.08
EPX	ND	ND	ND	PRG3	ND	ND	ND
FOXM1	0.54 ± 0.15	0.37 ± 0.15	0.52 ± 0.17	PRNP	0.69 ± 0.15	0.54 ± 0.18	0.44 ± 0.18
GLRX2	ND	ND	ND	PTGS1	0.46 ± 0.17	0.33 ± 0.24	0.36 ± 0.15
GPR156	ND	ND	ND	PTGS2	0.49 ± 0.16	0.48 ± 0.24	0.36 ± 0.14
<b>GPX1</b>	<b>0.98 ± 0.31</b>	<b>1.74 ± 0.45</b>	<b>0.70 ± 0.14</b>	PXDN	ND	ND	ND
GPX2	ND	ND	ND	PXDNL	ND	ND	ND
GPX3	0.59 ± 0.16	0.43 ± 0.17	0.42 ± 0.17	RNF7	0.51 ± 0.14	0.39 ± 0.14	0.36 ± 0.15
GPX4	0.50 ± 0.16	0.37 ± 0.15	0.40 ± 0.15	SCARA3	ND	ND	ND
GPX5	ND	ND	ND	SELS	4.51 ± 1.88	5.63 ± 2.24	4.15 ± 1.34
GPX6	ND	ND	ND	SEPP1	0.59 ± 0.15	0.42 ± 0.24	0.43 ± 0.18
<b>GPX7</b>	<b>1.29 ± 0.43</b>	<b>0.62 ± 0.22</b>	<b>1.14 ± 0.43</b>	SFTPD	0.49 ± 0.16	0.42 ± 0.24	0.37 ± 0.15
GSR	0.86 ± 0.28	0.81 ± 0.47	0.76 ± 0.28	SGK2	7.24 ± 2.7	7.38 ± 0.32	6.38 ± 2.62
GSS	0.32 ± 0.12	0.31 ± 0.12	0.36 ± 0.15	SIRT2	0.31 ± 0.12	0.29 ± 0.13	0.35 ± 0.14
GSTZ1	0.49 ± 0.17	0.52 ± 0.25	0.46 ± 0.14	<b>SOD1</b>	<b>1.20 ± 0.23</b>	<b>0.62 ± 0.20</b>	<b>0.54 ± 0.13</b>
GTF2I	0.33 ± 0.12	0.28 ± 0.09	0.26 ± 0.07	SOD2	0.48 ± 0.16	0.42 ± 0.24	0.48 ± 0.18
KRT1	ND	ND	ND	SOD3	ND	ND	ND
LPO	ND	ND	ND	SRXN1	0.28 ± 0.12	0.28 ± 0.13	0.36 ± 0.15
MBL2	ND	ND	ND	STK25	0.61 ± 0.15	0.51 ± 0.24	0.54 ± 0.16
MGST3	1.99 ± 0.99	1.73 ± 0.68	2.11 ± 1.23	TPO	ND	ND	ND
MPO	0.31 ± 0.12	0.24 ± 0.12	0.27 ± 0.08	<b>TTN</b>	<b>1.89 ± 0.35</b>	<b>0.86 ± 0.25</b>	<b>1.31 ± 0.61</b>
MPV17	0.85 ± 0.29	0.58 ± 0.25	0.71 ± 0.29	TXNDC2	ND	ND	ND
MSRA	0.63 ± 0.24	0.54 ± 0.24	0.62 ± 0.18	TXNRD1	0.41 ± 0.15	0.42 ± 0.24	0.36 ± 0.14
<b>MT3</b>	<b>1.51 ± 0.47</b>	<b>0.95 ± 0.34</b>	<b>0.94 ± 0.32</b>	TXNRD2	0.32 ± 0.12	0.41 ± 0.23	0.36 ± 0.15

**Table 2.** Screening analysis of antioxidant-related gene expression in peripheral blood mononuclear cells (PBMCs) of 24 drinkers. Values are presented as means ± SEM. ND not determined. Bold text indicates upregulated genes; that is, those with > 1.5-fold higher expression levels between any two of the three groups of drinkers.

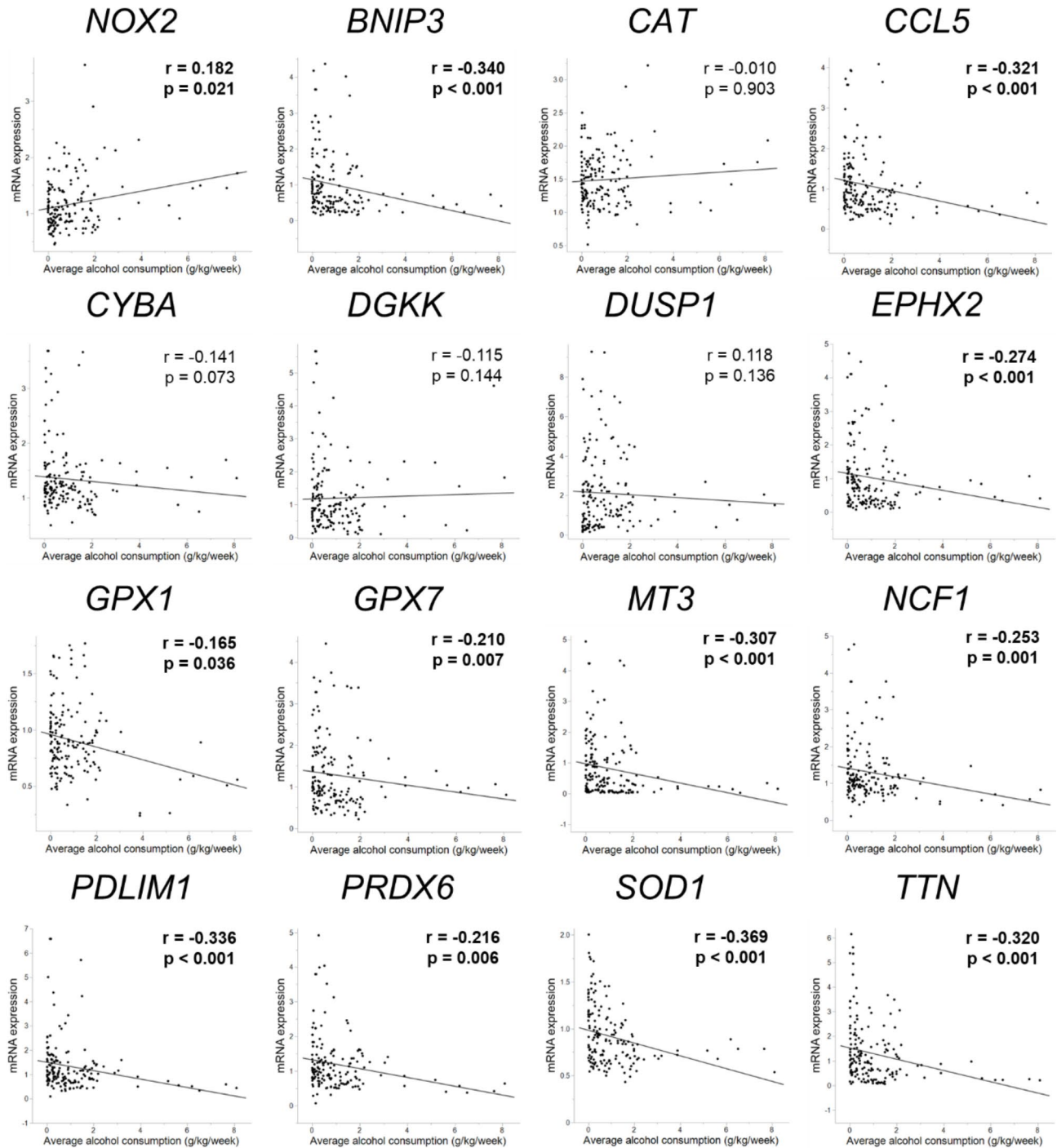
## Discussion

In this study, we examined the expression of several antioxidant-related genes in PBMCs in vitro and found that the antioxidant system was impaired by EtOH exposure at a young age. Next, we examined the expression of 84 antioxidant-related genes in PBMC samples from young male adults consuming alcohol early in life and found that the antioxidant system tended to be impaired according to the degree of alcohol consumption. Our results demonstrated that, among these 84 genes, *SOD1* is a powerful indicator of an impaired antioxidant system in peripheral immunity and can be a sensitive indicator of heavy alcohol consumption in young adulthood during EtOH exposure at a young age.



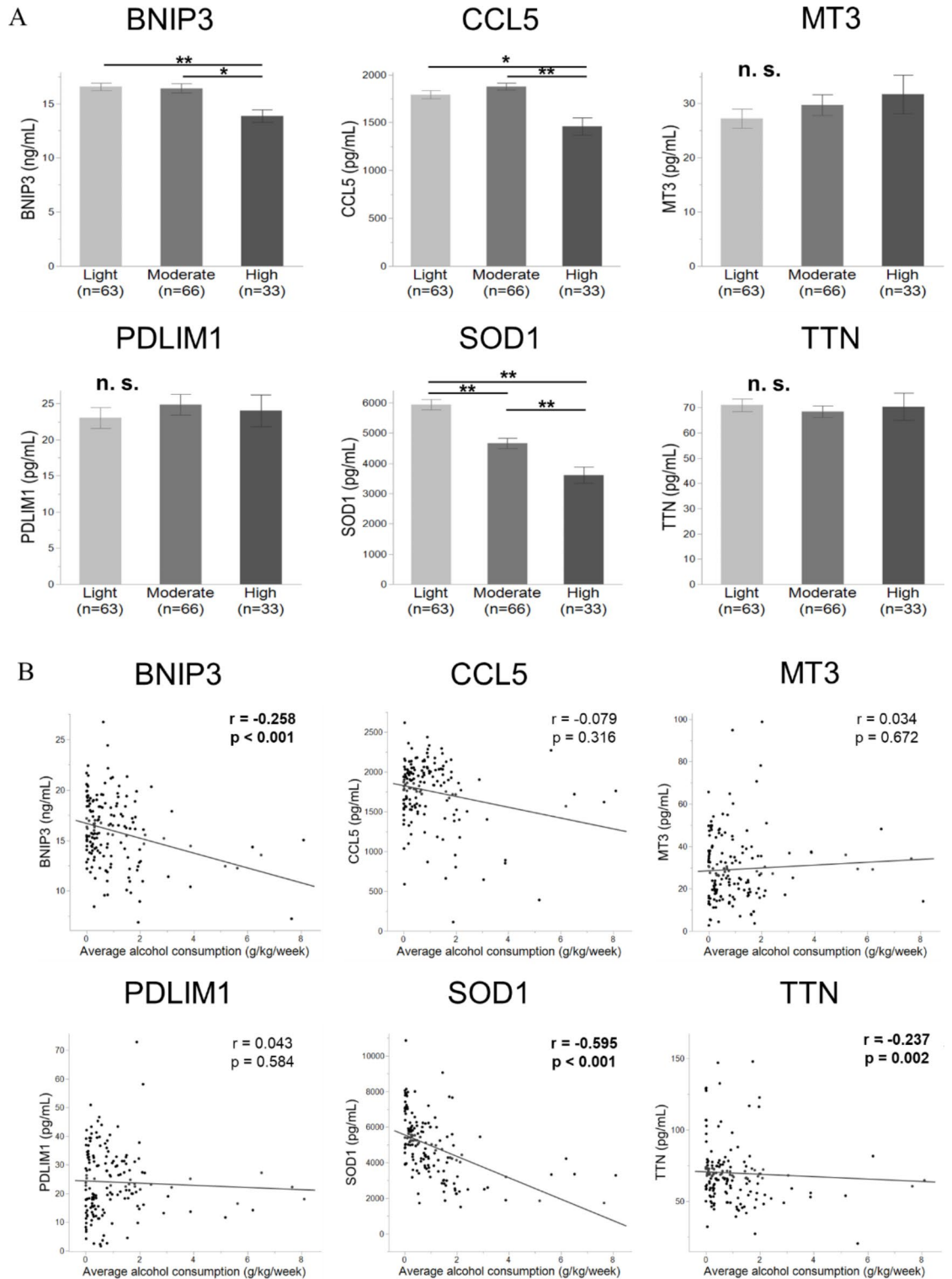


**Fig. 2.** mRNA expression levels of NOX2 and 15 antioxidant-related genes in 162 young adults. Students were divided into three alcohol-drinking groups (light:  $n = 63$ ; moderate:  $n = 66$ ; heavy:  $n = 33$ ) by average weekly alcohol consumption. Only the SOD1 expression level was significantly downregulated in the heavier drinking group compared to any two of the three drinking groups. The NOX2 expression level was significantly upregulated in the moderate- and heavy-drinking groups compared to the light group. Expression levels of BNIP3, CCL5, EPHX2, GPX1, GPX7, MT3, NCF1, PDLIM1, PRDX6, and TTN were significantly downregulated in the heavier-drinking group between at least two of the three groups. Data are expressed as the mean  $\pm$  SEM (\* $p < 0.05$ , \*\* $p < 0.001$ ; n. s., not significant; Wilcoxon Kruskal–Wallis test).



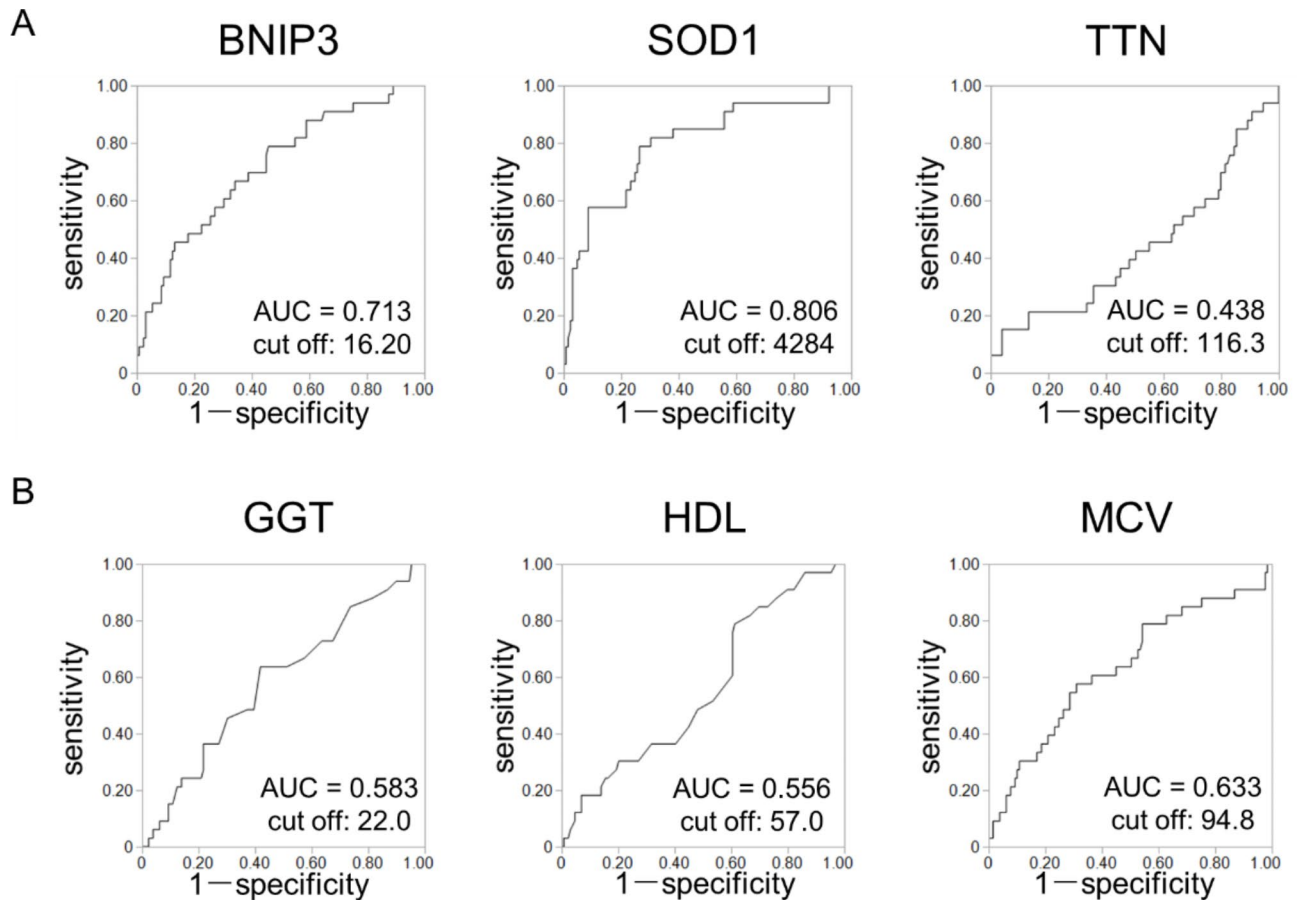
**Fig. 3.** Correlations between mRNA expression levels of NOX2 and 15 antioxidant-related genes and average weekly alcohol consumption in 162 young adults. NOX2 expression was positively correlated with average weekly alcohol consumption, and expression levels of 11 other mRNAs: SOD1, BNIP3, CCL5, EPHX2, GPX1, GPX7, MT3, NCF1, PDLIM1, PRDX6, and TTN were negatively correlated with average weekly alcohol consumption. Associations with  $p < 0.05$  are shown in bold.

The antioxidant system in peripheral immunity was found to be impaired by EtOH exposure at a young age in both in vitro and human studies. Notably, the system was impaired even with light alcohol consumption. Chronic, excessive alcohol consumption induces ROS production, which in turn induces OS in various organs and cells, including immune cells<sup>17,18</sup>, consequently impairing the immune system<sup>19</sup>. Alcohol-induced oxidative damage leads to the impairment of antioxidant activity, including those of SOD, CAT, and GPX<sup>20–23</sup>, and significantly affects the antioxidant-related expression of SOD and GPX in PBMCs<sup>6,13</sup>. However, consensus has not been reached on the decrease in antioxidant-related gene expression following acute EtOH exposure<sup>24</sup>.



**Fig. 4.** Plasma levels of six antioxidant-related proteins and correlations between the levels and average weekly alcohol consumption in 162 young adults. **(A)** The six applicable antioxidant-related gene proteins, namely those of the genes *BNIP3*, *CCL5*, *MT3*, *PDLIM1*, *SOD1*, and *TTN*, were selected. Only the *SOD1* plasma level was significantly downregulated in the heavier-drinking group between any two of the three drinking groups. Plasma levels of *BNIP3* and *CCL5* were significantly downregulated in the heavier-drinking group between at least two of the three groups (\* $p < 0.05$ , \*\* $p < 0.001$ ; n.s., not significant; Wilcoxon Kruskal–Wallis test). **(B)** Plasma levels of *BNIP3*, *SOD1*, and *TTN* were negatively correlated with average weekly alcohol consumption. Associations with  $p < 0.05$  are shown in bold.





**Fig. 5.** Selection of the sensitive indicators of high alcohol consumption at a young age. Indicators were assessed based on the area under curve (AUC) values calculated from receiver operating characteristic (ROC) curves. (A) ROC curves for plasma levels of antioxidant-related genes: *BNIP3*, *SOD1*, and *TTN*. (B) ROC curves for levels of alcohol-related parameters: gamma-glutamyl transferase (GGT), high-density lipoprotein cholesterol (HDL-C), and mean corpuscular volume (MCV). Of these 6 AUC values, the *SOD1* level had the highest AUC value of 0.806.

No reports have mentioned the decrease in antioxidant-related gene expression in peripheral immunity under EtOH exposure at a young age and damage to antioxidant enzymes due to light drinking. Light-to-moderate alcohol consumption increases the risk of developing cancer<sup>8,9</sup>, and a weekly alcohol consumption of 20 g/week adversely affects fetal growth<sup>25,26</sup>. Light-to-moderate alcohol consumption may also adversely affect the antioxidant system in peripheral immunity.

The reason why EtOH impairs PBMCs and reduces their antioxidant activity remains unknown. Originally, antioxidant enzymes are upregulated as a compensatory response to protect PBMCs from the damage of heavy alcohol consumption<sup>6</sup>. Alcohol intake alters the functions of both innate and adaptive immune cells, including PBMCs, and immune dysfunction has been associated with alcohol-induced end organ damage<sup>27</sup>. Chronic alcohol exposure impairs several immune cells, reduces the dysfunction of peripheral T cells, and causes the loss of peripheral B cells<sup>28</sup>. In this process, the activity of antioxidant enzymes such as SOD and GPx is impaired by decreasing copper/zinc or selenium deposits in the body<sup>26,29</sup>. Although similar reports on peripheral immune cells remain lacking, the antioxidant system in PBMCs may be impaired by a similar mechanism.

SOD is an enzyme that alternately catalyzes the dismutation of the superoxide radical into ordinary molecular oxygen and hydrogen peroxide, compared to the catalase and glutathione family<sup>30,31</sup>. Superoxide is a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Therefore, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen<sup>30</sup>. Lack of SOD decreases EtOH metabolism and is linked to enhanced OS<sup>29</sup>. Three forms of SOD are present in all mammals, including humans: SOD1 in the cells, SOD2 in the mitochondria, and SOD3 outside the cells<sup>30</sup>. SOD1, a key antioxidant marker in this study, also plays a protective role against OS and protects many of the cellular structures except the mitochondrial membrane. A lack of SOD1 results in accelerated aging, increased oxidative DNA damage, and promotes carcinogenesis, such as hepatocellular carcinoma<sup>32–34</sup>. In this study, the decreased level of SOD1 was the most sensitive indicator of EtOH exposure at a young age in human peripheral immunity. Although the reason for this is unclear, SOD1 itself is a major target of oxidative damage and also acts as a transcription factor regulating the OS response<sup>32</sup>. EtOH-induced oxidative damage primarily targets SOD1, and a reduced SOD1 mRNA level

has been observed in the liver tissues of EtOH-fed mice<sup>35</sup>. Therefore, SOD1 can be more susceptible to oxidative damage from EtOH exposure, unlike the catalase and glutathione family<sup>36</sup>. Furthermore, SOD1 in PBMCs may be more susceptible to oxidative damage from EtOH exposure in blood circulation. When considering non-alcohol-induced diseases, SOD1 can be pathogenic in various neurodegenerative diseases, and mutant SOD1 is especially associated with amyotrophic lateral sclerosis (ALS)<sup>30,33,37,38</sup>. However, alcohol consumption is not directly related to the pathology of ALS<sup>39</sup>, and the role of SOD1 may differ between ALS and alcohol-induced pathology.

In addition to SOD1, BNIP3, CCL5, MT3, PDLIM1, and TTN showed relatively strong negative correlations with the amount of alcohol consumed early in life. Acute EtOH treatment increases BNIP3 expression in the liver<sup>40</sup>, and BNIP3-dependent mitophagy limits ROS production and promotes cell survival in cardiomyocytes, tumor cells, and hepatocytes<sup>41</sup>. BNIP3 is also expressed in PBMCs<sup>42</sup>, and its deletion increases cellular ROS<sup>43</sup>. CCL5 is a member of the proinflammatory cytokine superfamily and the CC chemokine family and is produced by PBMCs, especially monocytes<sup>44</sup>. Patients with alcoholic liver disease have increased hepatic CCL5 levels<sup>45</sup>, and EtOH augments CCL5 expression in vivo<sup>46</sup>. CCL5, via GPX1 activation, protects hepatocytes from ROS damage<sup>47</sup>. MT has a high affinity for zinc<sup>48</sup>, and zinc sulfate has antioxidant effects on acute EtOH-induced oxidative damage<sup>49</sup>. MT can scavenge reactive species, and MT3 exerts a greater protective effect against DNA degradation from oxidative damage than that of MT1/2<sup>50</sup>. In peripheral immunity, activated MT3 impairs antibacterial immunity<sup>51</sup>; however, the expression and detailed role of MT3 in PBMCs remain unknown.

PDLIM1 has several important roles in cytoskeletal organization, neuronal signaling, and organ development. It also plays roles in cell proliferation and metastasis during tumor initiation and progression when expressed in PBMCs<sup>52,53</sup>. PDLIM1 has an antioxidant effect on neuro- and cardio-degenerative diseases<sup>53</sup>. TTN has an accepted role in mechanical protein in muscle cells, such as passive force production and stabilization of sarcomeres, and has controversial contributions to residual force enhancement, passive force enhancement, energetics, and work production in shortening muscles<sup>54</sup>. TTN is expressed in human T and B lymphocytes as a critical and versatile housekeeping regulator of T lymphocyte trafficking<sup>55</sup>. Chronic alcohol consumption reduces the TTN level and promotes muscle atrophy<sup>56</sup>. ROS modify TTN in cardiomyocytes<sup>57</sup>; however, the reduction of ROS by TTN is unclear.

These reports suggest that all five antioxidant-related genes contribute to the reduction of ROS damage via antioxidant reactions. However, the detailed mechanisms have not been elucidated, and these associations with EtOH-induced OS in peripheral immunity are unknown and must be elucidated in the future. These five genes may also be targets of oxidative damage via unknown mechanisms, similar to SOD1, which is a novel finding in this study.

In this study, SOD1 at the plasma and mRNA levels was most negatively correlated with the amount of alcohol consumed. Plasma SOD1 levels are significant indicators of cutaneous leishmaniasis and ALS<sup>38,58</sup>; however, the change in SOD1 plasma levels as well as in the BNIP3, CCL5, MT3, PDLIM1, and TTN plasma levels due to EtOH exposure remains unknown. Possibly, SOD1, BNIP3, CCL5, MT3, PDLIM1, and TTN are secreted from not only PBMCs but also other cells such as vascular endothelial, muscle, nerve, and cancer cells, which may affect their plasma levels. Among the six antioxidant-related genes, SOD1 may be secreted at greater levels from PBMCs under early EtOH exposure. Furthermore, our results indicate that, compared to GGT, HDL, and MCV, the SOD1 level is a more sensitive indicator of heavy alcohol consumption in young male adults. The levels of GGT, HDL, and MCV are higher in heavy drinkers among adults and are clinically used as significant indicators of heavy alcohol consumption<sup>59,60</sup>. Although the reason for the above results is unclear, intracellular SOD1 in PBMCs, a major target of oxidative damage<sup>23,25</sup>, may be impaired earlier by EtOH exposure, and the impairment may be faster than GGT, HDL, and red blood cells, which are gradually impaired by exposure to various mechanisms.

In this study, we demonstrated that SOD1 is an important indicator of the downregulation of the antioxidant system in peripheral immunity under EtOH exposure at a young age and among heavy alcohol consumers at a young age. Human PBMC samples are required to verify the results of decreased antioxidant-related gene expression obtained from in vitro PBMCs. To eliminate errors induced by sex differences, we standardized our investigation to male PBMC samples; however, this was a limitation. In the future, a validation study including both male and female PBMC and plasma samples will be necessary for clinical applications of our findings. As females are more susceptible to alcohol-induced liver damage than males<sup>22</sup>, differential SOD1 expression by sex may also be observed in susceptibility to liver damage. Therefore, our results should only be generalized following separate analyses of males and females to avoid sex biases. Furthermore, we excluded subjects with smoking habits and several diseases that may affect OS, and focused on examining the relationship between early alcohol drinking and OS; this was a limited study. The relationship between alcohol drinking and OS should be examined from various perspectives, including smoking and these diseases, which will be a future task.

In conclusion, the antioxidant system in peripheral immunity tends to be impaired by EtOH exposure at a young age. Among several antioxidant-related genes, SOD1 is a powerful indicator of antioxidant system impairment and can be a sensitive indicator of heavy alcohol consumption in young adulthood. Our data suggest that even a small amount of early alcohol consumption downregulates the antioxidant system, which can be a novel risk factor for OS-induced diseases. Our study introduces the prospect of preventing immunity-related disorders caused by an increase in OS through guidance on appropriate alcohol drinking in young adulthood and will contribute to the early detection of excessive drinkers among young adults.

## Materials and methods

### University students (undergraduates and graduates)

From March 2021 to June 2022, 197 male university students (young adults aged 22–29 years,  $n = 162$ ; adolescents aged 18–19:  $n = 35$ ) who underwent health checkups overnight at a university in Japan were enrolled in the

study. According to Erikson's psychosocial theory, adolescence (12–18 years old) and young adulthood (19–29 years old) are divided at age 18–19<sup>61,62</sup>. In this study, first-year students were 18–19 years old and integrated as “adolescents”. All the participants were Japanese and provided informed consent to participate in this study. To avoid the effects of OS caused by smoking and several diseases, the participants were non-smokers and had no symptoms of acute infectious diseases, history of malignant tumors, or inflammatory or autoimmune diseases. Female students were excluded to eliminate differences in susceptibility to alcohol exposure between the sexes; notably, they have been found to consume less alcohol than males at our university<sup>63,64</sup>. In Japan, drinking alcohol under the age of 20 is prohibited by law, and all adolescents are non-drinkers. Although the recent rate of alcohol drinking among adolescents in Japan is about 2%<sup>65</sup>, in this study, the non-drinkers had no alcohol-drinking habits through our detailed interviews. This study was approved by the Ethics Committees of the University of Tokyo Clinical Research Review Board (Protocol No. 18-277). The study was performed in accordance with the Declaration of Helsinki guidelines and regulations.

### Alcohol consumption

The health checkup included the completion of a standard questionnaire, a physical examination, and biochemical tests. The questionnaire, which was created for this study, evaluated alcohol drinking habits, and the average daily consumption per kg of body weight (BW) (g/kg/day) and frequency (days/week) of alcohol consumption (amount of pure alcohol) were recorded<sup>66,67</sup>. To obtain accurate information on alcohol consumption, we carefully explained to the participants that self-reporting would not be detrimental to them. In this study, the daily consumption was defined as the average drinking amount only on drinking days, and weekly consumption (g/kg/week) was calculated by multiplying the frequency by the daily consumption. Young adults who were exposed to EtOH early were divided into three alcohol-drinking groups: light, moderate, and heavy, according to their average weekly alcohol consumption, compared to adolescent non-drinkers. The non-drinkers were also non-smokers, and had no comorbidities, no history of alcohol consumption, no family history of alcohol/drug use, or no special medications, which may affect OS.

Standard definitions of alcohol consumption vary according to the country<sup>68</sup>. First, in this study, “heavy-drinking” group was defined based on the alcohol consumption of >100 g/week, with a higher mortality rate<sup>10,69</sup>, whereas alcohol consumption of ≤100 g/week was considered “light-to-moderate drinking”<sup>69</sup>. The alcohol-drinking group of ≤100 g/week was dichotomized into 20–100 g/week (moderate-drinking) and <20 g/week (light-drinking). Next, individual average weekly alcohol consumption (g/kg/week) was calculated. Furthermore, to recalculate the two cutoff values, 20 and 100 (g/week) in g/kg/week, they were divided by the mean of the participants' BW, 62.8 kg; its standard error was 0.6. Finally, they were divided into the following three groups: light (<0.31 g/kg/week), moderate (0.31–1.59 g/kg/week), and heavy (>1.59 g/kg/week).

### Clinical parameters

Body height, BW, body mass index (BMI), systolic blood pressure, and diastolic blood pressure (DBP) were measured on the day of the health checkup. Biochemical parameters included the levels of AST, ALT, GGT, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), uric acid, creatinine, C-reactive protein, and fasting blood glucose (FBS) and platelet count and MCV.

### PBMC samples

PBMCs were separated from the whole blood samples of 197 university students using a Ficoll kit using 8 mL Vacutainer Cell Processing tubes (BD Biosciences; Franklin Lakes, NJ, United States), as described in our previous study<sup>12</sup>. All participants provided informed consent for PBMC collection. In brief, blood samples were processed within 4 h of blood collection by collecting the PBMC-containing upper phase and washing with ice-cold phosphate buffered saline (PBS). PBMCs were either frozen for later experiments or used directly for the enrichment of the erythroblast population.

### Cell culture

PBMCs were obtained from healthy, non-drinking university students (non-drinkers) and were suspended in 10 mL RPMI 1640 medium (Merck, Darmstadt, Germany) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine, and 10% fetal bovine serum after two washes (1500 g, 5 min, 23 °C). They were adjusted to  $5.0 \times 10^5$ /mL and incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub> for 24 h in 6-well plates with RPMI 1640 medium ( $1.0 \times 10^6$  cells/well) as previously reported<sup>70,71</sup>. At 70–80% confluence, PBMCs were treated with or without (used as a control) various concentrations of EtOH (6.25, 12.5, 25, 50, and 100 mM) for 24–72 h. According to the Widmark formula, EtOH 6.25 mM was equivalent to 0.18 g/kg/day, and 100 mM to 2.93 g/kg/day, in human daily alcohol consumption<sup>72</sup>; as shown in Table 1, the range of EtOH 6.25–100 mM corresponded to moderate-heavy drinking. Thereafter, PBMCs were withdrawn, followed by two or three washes with PBS, and were directly used or frozen for reactive oxygen species (ROS) detection, cell viability, and examination of antioxidant-related gene expression. EtOH was obtained from Wako Industries (Osaka, Japan).

### ROS detection

Intercellular ROS formation was assessed using a DCFDA/H2DCFDA-Cellular ROS Assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. DCFDA is a cell-permeable non-fluorescent probe that diffuses into the cytoplasm and then forms a non-fluorescent moiety through deacetylation by cellular esterase. It is oxidized by cellular ROS to form the fluorescent product DCF<sup>73</sup>.

PBMCs were cultured in gelatin-coated 96-well black plates ( $5.0 \times 10^4$  cells/well) and 6-well plates ( $2.0 \times 10^6$  cells/well) overnight and treated with or without (used as a control) various concentrations of EtOH (6.25, 12.5, 25, 50, and 100 mM) for 24–72 h at 37 °C in an atmosphere containing 5% CO<sub>2</sub>, followed by incubation with 20

$\mu\text{M}$  DCFDA at 37 °C in the dark for 45 min. After two or three washes with PBS buffer, the fluorescence intensity of cellular ROS in the 96-well black plates was measured at 485/535 nm using a DTX plate reader (Beckman Coulter, Fullerton, CA, USA). ROS levels are expressed as the relative fold change in DCF fluorescence units with respect to the control.

### Cell viability

Cell viability was evaluated using an XTT Cell Viability Assay kit (Biological Industries, Cromwell, CT, USA) according to the manufacturer's instructions. PBMCs were cultured in gelatin-coated 96-well plates ( $5.0 \times 10^4$  cells/well) with or without (used as a control) various concentrations of EtOH (6.25, 12.5, 25, 50, and 100 mM) for 24–72 h at 37 °C with 5%  $\text{CO}_2$ . After incubation, the medium was discarded, and 100  $\mu\text{L}$  of fresh medium was added to each well. Then, 50  $\mu\text{L}$  of the activated XTT solution was added, and samples were incubated for 2 h at 37 °C. The absorbance ( $\lambda = 450 \text{ nm}$ ) was measured using the above DTX plate reader. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability \%} = (\text{Absorbance of sample} - \text{Blank}) / (\text{Absorbance of control} - \text{Blank}) \times 100$$

### Quantitative real-time polymerase chain reaction

mRNA was extracted from PBMCs using the RNeasy Micro Kit (Qiagen, Hilden, Germany), and complementary DNA was synthesized using the RT2 First Strand Kit (Qiagen) as previously reported<sup>12</sup>. First, TaqMan™ Array, Human Antioxidant Mechanisms, Fast 96-well (Thermo Fisher Scientific, Waltham, MA, USA) was used to examine the expression levels of 84 antioxidant-related genes in 24 young adults (light: moderate: heavy = 8: 8: 8) who were randomly selected. A list of the genes examined using this array is shown in Table S2. Gene expression analysis was performed using TaqMan PCR (Thermo Fisher Scientific). Next, the expression levels of the identified genes in all 197 students were determined using TaqMan PCR. The same PCR primers and probes as those used for the above array were used. The other specific PCR primer and probe were for nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2; Hs00166163\_m1, another member of the NOx families that produce ROS in immune cells (including PBMCs), was used to evaluate the degree of ROS production in human PBMC samples in this study<sup>74</sup>. Furthermore, in vitro expression changes of five genes (SOD1, SOD2, GPX1, catalase [CAT], and NOX2) in PBMCs were examined with or without treatments of various concentrations of EtOH for 24–72 h. Expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the relative expression levels were calculated.

### Measurement of antioxidant markers in plasma

Whole blood samples were obtained from all 197 students and centrifuged at 1000 g for 10 min. Plasma samples were separated and stored at  $-20$  °C until analyzed. The plasma levels of six antioxidant-related proteins, Bcl-2/adenovirus E1B 19-kDa protein-interacting protein (BNIP) 3, CC chemokine ligand (CCL) 5, metallothionein (MT) 3, PDZ and LIM domain (PDLIM) 1, SOD1, and titin (TTN), were measured using a commercial ELISA kit, according to the manufacturer's instructions. The plasma levels were determined and validated using commercially available ELISA kits (Table S4).

### Statistical analysis

Data were analyzed using JMP 17 software (SAS Institute Japan, Tokyo, Japan).  $p < 0.05$  was considered statistically significant. The sample size required for this study was calculated using G\*power calculator 3.1.9.7 software (Heinrich-Heine-Universität, Düsseldorf, Germany) (effect size = 0.65, alpha error = 0.05, power = 0.80).<sup>75</sup> For in vitro absorbance and fluorescence analyses and mRNA expression determined using TaqMan PCR, data were analyzed using an unpaired Student's *t*-test. Nonparametric data were analyzed using Wilcoxon Kruskal–Wallis test. Correlations among alcohol consumption in alcohol drinkers, gene expression, and plasma levels were examined using Spearman's rank correlation method. Age-related changes in non-drinkers were assessed in the above analyses between adolescents and young adults consuming light alcohol. Data are expressed as the mean  $\pm$  standard error of the mean (SEM) unless stated otherwise.

### Data availability

Data is provided within the manuscript or supplementary information files.

Received: 7 April 2024; Accepted: 10 October 2024

Published online: 23 October 2024

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## Acknowledgements

We thank Ms. Seiko Shinzawa (Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Japan) for providing technical assistance. We thank the staff of the Department of Pathology, Graduate School of Medicine, University of Tokyo, for the processing and pathological examination of the specimens. This research was supported by a Health Sciences Research Grant from the Ministry of Health, Labor, and Welfare of Japan (Research on Hepatitis) and a Grant from the Japan Agency for Medical Research and Development (AMED) under Grant Number JP23fk0210090. No additional external funding was received for the study. The funders played no role in the study design, data collection, analysis, decision to publish, or manuscript preparation.

## Author contributions

A.K., K.M., and Y.I. contributed to the study design, data acquisition, data analysis, and interpretation, and manuscript drafting. S.Y., T.T., and K.K. participated in critical revision of the manuscript. M.F. participated in the analysis and interpretation of the data, drafting and critical revision of the manuscript, and supervision. All

authors have reviewed and approved the final version of the manuscript.

## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-76084-8>.

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