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ORIGINAL ARTICLE

Comprehensive analysis of peripheral blood free amino acids in MASLD: the impact of glycine-serine-threonine metabolism

Masaaki Mino[1] · Eiji Kakazu[1,2,6] · Akitoshi Sano[2] · Mio Tsuruoka[2] · Hiroko Matsubara[3] · Keisuke Kakisaka[4] · Takayuki Kogure[5] · Katsunori Sekine[1] · Yoshihiko Aoki[1] · Masatoshi Imamura[1] · Michitaka Matsuda[6] · Taiji Yamazoe[6] · Taizo Mori[6] · Sachiyo Yoshio[6] · Jun Inoue[2] · Atsushi Masamune[2] · Tatsuya Kanto[1,6]

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Abstract

Little is known about how blood free amino acids (FAAs) change in metabolic dysfunction-associated steatotic liver disease (MASLD). This study aims to identify the imbalance of FAAs in MASLD and explore its correction as a potential therapeutic target. We analyzed plasma FAAs data from 23,036 individuals with steatosis information from a biobank in Japan, and 310 patients with MASLD were enrolled. According to diagnostic criteria for steatotic liver disease (SLD) or cardiometabolic criteria (CC), we divided the subjects into five groups: MASLD, metabolic dysfunction and alcohol-associated liver disease (MetALD), CC-SLD-, CC + SLD-, and CC-SLD + . Twenty FAAs were compared among these groups and among MASLD patients with pathological information. Among the 20 FAAs, the levels of 16 FAAs increased in CC + SLD- according to the number of matches with CC items associated with insulin resistance (IR). Steatosis enhanced most of these changes but serine (Ser) and threonine (Thr) were unaffected. Glycine (Gly), Ser and Thr were significantly decreased in patients according to steatosis grade. We investigated the association between these FAAs imbalances and pathogenesis using MASLD mouse models. In mice fed a high-fat, fructose, and cholesterol (FFC) diet, metabolomics and RNA sequencing analyses indicated that abnormality in Gly, Ser, and Thr metabolism in the liver was associated with mitochondrial dysfunction and enhanced glycolysis via pyruvate. High-Gly, Ser, and Thr diet ameliorated pathogenesis of MASLD in leptin-deficient mice. Most FAAs increase due to cardiometabolic abnormalities, particularly IR. However, interventions targeting the metabolism of Gly, Ser, and Thr have the potential to improve MASLD.

Keywords SLD · MetALD · MASH · NASH · Cardiometabolic criteria

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✉ Eiji Kakazu
kakazu@coral.ocn.ne.jp

[1] Department of Gastroenterology and Hepatology, Kohnodai Hospital, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa, Chiba 272-8516, Japan

[2] Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

[3] Department of Biobank, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi, Japan

[4] Division of Gastroenterology and Hepatology, Iwate Medical University School of Medicine, Yahaba, Iwate, Japan

[5] Division of Gastroenterology Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan

[6] Department of Liver Diseases, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan

Abbreviations

AAAs	Aromatic amino acids
AAs	Amino acids
AIN-93G	American Institute of Nutrition 1993 Growth
Ala	Alanine
Alb	Albumin
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
Arg	Arginine
Asn	Asparagine
AST	Aspartate aminotransferase
BCAAs	Branched chain amino acids
BMI	Body mass index
BP	Blood pressure
BW	Body weight
CC	Cardiometabolic criteria

CC-SLD-	Cardiometabolic criteria negative and steatotic liver disease negative
CC + SLD-	Cardiometabolic criteria positive and steatotic liver disease negative
CC-SLD +	Cardiometabolic criteria negative and steatotic liver disease positive
CE-TOFMS	Capillary electrophoresis-time-of-flight mass spectrometry
Cys	Cysteine
ER	Endoplasmic reticulum
FAAs	Free amino acids
FBG	Fasting blood glucose
FFC	High-fat, fructose and cholesterol
FIB-4	Fibrosis-4 index
FLI	Fatty liver index
GGTP	Gamma-glutamyl transpeptidase
GLC	Glucose
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
H-GST	High glycine, serine and threonine
HE	Hematoxylin and eosin
His	Histidine
HSI	Hepatic steatosis index
Ile	Isoleucine
IR	Insulin resistance
KET	Ketone body
Leu	Leucine
Lys	Lysine
MASH	Metabolic dysfunction-associated steatohepatitis
MASL	Metabolic dysfunction-associated steatotic liver
MASLD	Metabolic dysfunction-associated steatotic liver disease
Met	Methionine
MetALD	Metabolic dysfunction and alcohol-associated liver disease
NAS	NAFLD activity score
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OBE	Obesity
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
SLD	Steatotic liver disease
T-cho	Total cholesterol
TG	Triglyceride
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UA	Uric acid
Val	Valine
WC	Waist circumference

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common global hepatic disorder and the number of patients with NAFLD has increased drastically. An estimated 38% of the global adult population was affected between 2016 and 2019 (Younossi et al. 2023). Recently, metabolic dysfunction-associated steatotic liver disease (MASLD), has been redefined in place of NAFLD or nonalcoholic steatohepatitis (NASH) with worldwide consensus by Delphi method (Rinella et al. 2023). MASLD focuses on the metabolic abnormalities in patients with steatosis, and is diagnosed on the basis of the presence of steatosis and five metabolic dysregulation [obesity (OBE), glucose (GLC), hypertension (HT), triglyceride (TG) and HDL] as shown in the cardiometabolic criteria (CC). There is a significant overlap between patients categorized as having MASLD and NAFLD (Hagström et al. 2024; Lee et al. 2024). In addition, a new category, outside pure MASLD, termed metabolic dysfunction and alcohol-associated liver disease (MetALD), was selected to describe those with MASLD who consume greater amounts of alcohol.

Various imbalances of metabolites are observed, as well as glucose and lipids in MASLD or MetALD. That induces cell stress (Venkatesan et al. 2023) in hepatocytes as lipotoxicity and then progresses the pathogenesis (Parthasarathy et al. 2020). Although glycemic and lipidemic abnormalities are considered in CC to diagnose MASLD, it is unknown how an imbalance of free amino acids (FAAs) in peripheral blood is observed. Previous studies reported that branched chain amino acids (BCAAs) and aromatic amino acids (AAAs), especially tyrosine (Tyr) was higher in plasma of NAFLD (Yamakado et al. 2017; Gaggini et al. 2018) and these profiles were the risk of developing diabetes (Wang et al. 2011). We have recently elucidated the differences in FAAs imbalance observed in chronic liver disease and cirrhosis across different etiologies (Mino et al. 2024). Besides, there are many studies on efficacy of amino acids (AAs) formulations for cirrhosis regardless of the etiology (Marchesini et al. 2003; Muto et al. 2005). In basic research, it has become clear that FAAs, especially glycine (Gly), serine (Ser), threonine (Thr) and methionine (Met) have a role as epigenomic modulators through one-carbon metabolism to maintain healthy condition (Ducker and Rabinowitz 2017). Met and Tyr are associated with the pathogenesis of NAFLD via oxidative response to lipotoxicity (Sano et al. 2021; Sáenz de Urturi et al. 2022). Therefore, it is important to understand how FAAs changes occur in peripheral blood according to the disease progression of MASLD. Here, we elucidated the imbalance of FAAs in MASLD using a database of the Japanese

general population and patients. Furthermore, we investigated whether dietary intervention with amino acid-balanced diets could improve the pathogenesis of MASLD mouse model.

Methods

Disclosure of ethical statements

The study was carried out in accordance with both the Declarations of Helsinki and Istanbul. The study protocol was approved by the institutional review board of National Center for Global Health and Medicine (NCGM-S-004343-02). This study is a retrospective observational study for patients with MASLD, carried out using the opt-out method at four hospitals: Kohnodai Hospital, Tohoku University Hospital, Iwate Medical University Hospital, and Tohoku Medical and Pharmaceutical University Hospital. Additionally, data of general adults were obtained from the Tohoku Medical Megabank Project (research number: 2023-0012). The murine studies and use were approved by the animal experiment committee of National Center for Global Health and Medicine (2023-A073) and were conducted according to the Guidelines for Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Subjects: general adults

We obtained the data of 28,823 general population above 18 years old from a biobank: Tohoku Medical Megabank Organization in Japan. The data include medical, lifestyle, laboratory, and metabolome information, with medical and lifestyle details collected via self-administered questionnaires. We excluded 5,787 subjects; missing data and a history of 12 types of malignant neoplasms (stomach, colon, lung, liver, kidney, pancreas, skin, breast, testicular, prostate, brain, leukemia, malignant lymphoma, multiple myeloma, ovarian, cervical and uterine).

We analyzed 23,036 subjects who responded to the question, 'Have you been, or have you ever been diagnosed with fatty liver by a physician?' If the answer is 'Yes', the subject was defined as SLD. All subjects have data on CC to diagnose MASLD (Rinella et al. 2023). We categorized into the following five groups based on CC for adult, i; OBE: body mass index (BMI) $\geq$ 23 kg/m^2 or waist circumstance > 94 cm for male and 80 cm for female, ii; GLC: Fasting serum glucose $\geq$ 5.6 mmol/L [100 mg/dL] or HbA1c $\geq$ 5.7% [39 mmol/L] or type 2 diabetes or treatment for type 2 diabetes (we did not included criterion; 2-h post-load glucose levels $\geq$ 7.8 mmol/L [$\geq$ 140 mg/dL]), iii; HT: BP $\geq$ 130/85 mmHg or specific antihypertensive drug treatment, iv; TG: Plasma TG $\geq$ 1.70 mmol/L [150 mg/dL] or lipid lowering treatment, v; HDL: Plasma HDL-cho $\leq$ 1.0 mmol/L [40 mg/dL] for male and $\leq$ 1.3 mmol/L [50 mg/dL] for female or lipid lowering treatment (Supplemental Table S1). If the subjects fulfilled at least one criterion, they were defined as CC positive (CC+). Otherwise, they were defined as CC negative (CC-). In this study, the number of matches with CC was defined as the CC score, which ranged from 1 to 5. The subjects who met the criteria for both SLD and CC were categorized into either MASLD or MetALD, including alcoholic liver disease (ALD) based on their ethanol consumption of over 40 g per day. Ethanol consumption was calculated from six types of alcoholic beverage (beer, whisky, wine, sake, shochu and chuhai) based on a questionnaire survey. The ethanol concentrations of these beverages were 5% 40%, 12%, 15%, 12.5% and 6%, respectively. The change of body weight (BW) for a year was calculated by the information of questionnaire. On SLD negative (SLD-) subjects, sex and age were matched to MASLD (Supplemental Figure S1). The algorithm applied was 1:2 (CC-SLD-) and 1:10 (CC + SLD-) nearest available matching with a $\pm$ 0.2 caliper, and without replacement. We finally divided subjects into five groups (MASLD, MetALD, CC-SLD+, CC + SLD- and SLD-CC-). The flow chart of selection of subjects in this study was shown in Supplemental Figure S2. Hepatic steatosis index (HIS) and fatty liver index (FLI), scoring systems for assessing fatty liver, were calculated as follows:

- HIS (Lee et al. 2010) = 8 × (ALT/AST ratio) + BMI + (an additional 2 points were added for female participants and 2 points for the presence of type 2 diabetes).
- FLI (Bedogni et al. 2006) = $(e^{0.953*\ln(TG)+0.139*BMI+0.718*\ln(GGT)+0.053*WC-15.745})/(1+e.^{0.953*\ln(TG)+0.139*BMI+0.718*\ln(GGT)+0.053*WC-15.745}) \times 100$

Subjects: MASLD patients

Of 512 SLD patients, 310 MASLD patients diagnosed by biopsy (n = 170) or images (n = 140) were also enrolled from 4 hospitals after exclusion criteria (Supplemental Figure S2) to investigate the change of FAAs according to disease progression. We excluded the following cases: those considered to have ALD or MetALD due to ethanol consumption of 30 g or more per day (n = 54), cases with missing data (n = 121), individuals with serum creatinine levels greater than 2 mg/dL, drug-induced cases (n = 14), and those not meeting the cardiometabolic criteria (n = 12). The pathological features of patients diagnosed by biopsy (n = 170) were evaluated by the NAFLD activity score (NAS) (Brunt et al. 2011) in a blinded manner by an experienced hepatologist. Briefly, NAS was composed of four parameters: steatosis, inflammation, ballooning, and fibrosis. Fibrosis stages were divided into four groups: fibrosis stages 0 and

1 (F0-1), and fibrosis stages 2, 3, and 4 (F2, F3, and F4, respectively). Metabolic dysfunction-associated steatohepatitis (MASH) was defined as patients with a fibrosis stage ≥ 2 or a NAS ≥ 4 (Hjelkrem et al. 2011; Dulai et al. 2017), and early MASH was defined as the absence of fibrosis (F0) or mild fibrosis (F1), and all others were defined as metabolic dysfunction-associated steatotic liver (MASL). HOMA-IR of 211 patients was calculated to evaluate insulin resistance using the formula: HOMA-IR = fasting blood glucose (mg/dL) × fasting blood insulin (μU/mL) / 405.

Blood test and measuring FAAs

FAAs data of general population was abstracted from metabolomics data measured quantitatively by nuclear magnetic resonance (NMR) spectroscopy in Tohoku Medical Megabank Organization. The methods were described previous report (Koshiba et al. 2018). For patients, The blood test was performed in the early morning to obtain a fasted state sample, and the sample for the FAAs was collected in the EDTA tube and trichloroacetic acid was added to plasma or serum to a final concentration of 2.5%. The samples were then placed on ice for 15 min followed by centrifugation to remove precipitated proteins. The extracts were then analyzed for the amino acid content by HPLC/ninhydrin with an amino acid analyzer; L-8900 (HITACHI, Japan) or HPLC / electrospray mass spectrometry (HPLC/ESI-MS) (Shimbo et al. 2010) under contract to SRL, Inc., Japan. Twenty of 39 FAAs could be analyzed in this study.

Mice experiments

Six-week male C57BL/6 wild-type mice and five-week male C57BL/6 J ob/ob mice were purchased from Japan SLC, Inc or Jackson Laboratory Japan. Mice were housed in standard pathogen-free facilities with 12 h day-night circadian cycles and unrestricted access to food and water. Fifty-week-old wild-type mice (n = 10) were fed with a normal chow diet (NC, CE-2 [CLEA, Japan]) or a high–saturated fat, high-fructose and high cholesterol (FFC) diet (Charlton et al. 2011) for 25 weeks. The sample size for each group was five. The metabolites in liver were measured after 4 h fasting by capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS). For RNA sequencing analyses, total RNA is extracted from liver tissue using the RNeasy Mini Kit (QIAGEN, CA, USA) by homogenizing the sample in RLT buffer, adding ethanol, passing the mixture through a spin column, washing with RW1 and RPE buffers, and eluting with RNase-free water; RNA quality is then assessed using a NanoVue Plus (GE Healthcare, NJ, USA) spectrophotometer. Detailed methods for CE-TOFMS and RNA sequencing analyses are described in the Supplemental Methods. To compare FAAs in the liver, fifty-week-old male C57Bl/6 J wild-type mice were fed each diet (NC, n = 5; FFC, n = 5) for 20 weeks. Eight-week-old male C57Bl/6 J wild-type mice were fed each diet (NC, n = 5; FFC, n = 5) for 20 weeks and 2 weeks, respectively. Six-week-old ob/ob mice were fed either AAs defined American Institute of Nutrition 1993 Growth (AIN-93G, n = 7) or an AIN-93G based diet enriched with high levels of Gly, Ser and Thr (H-GST, n = 7) for three weeks (Supplemental Table S2). These diets were designed to match the total amount of AAs by adjusting the contents of BCAAs, Glu and Tyr in place of Thr, Ser and Gly. The components except AAs are consistent among chows. Mice were fasted for 4 h and then sacrificed after sedation under isoflurane (Mylan Pharmaceutical Co., Japan) to collect liver and plasma samples. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol and TGs were measured by SPOT-CHEM EZ SP-4430 (Arkray, Japan). For histological analysis, the liver tissues were fixed and embedded in paraffin for Hematoxylin and Eosin (HE) and masson trichrome (MT) staining.

Statistical analysis

Differences of the biochemical data and the levels of FAAs among groups were analyzed with ANOVA, and subsequent multiple comparisons were performed using the Dunnett's post-hoc procedure. The student's t-test was used for the comparison between two groups. Data are expressed as mean ± SD. All P value of < 0.05 was considered statistically significant. All statistical analyses were performed with JMP® Pro 17 and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). The principal component analysis (PCA) plots of the annotated metabolomics data were generated using MetaboAnalyst software 6.0 (https://www.metaboanalyst.ca/) with log-transformed intensities and auto-scaling for normalization. Metabolite enrichment analysis was performed exclusively on annotated metabolites (HMDB ID) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) library and enrichment pathway tool in MetaboAnalyst software 6.0. Detailed information on the PCA analysis and pathway enrichment analysis performed using the MetaboAnalyst software is provided in the supplementary information and supplementary materials.

Results

Characteristics of the enrolled general adults and patients

In this study, we evaluated 23,036 general adults, identifying 1,220 as having SLD and 21,816 as not having SLD, using a questionnaire. Based on the CC, and after matching for age and sex (Supplemental Figure S1), we categorized subjects into five groups: CC-SLD- (n = 1756), CC + SLD- (n = 8900), MASLD (n = 890), MetALD (n = 262) and CC-SLD + (n = 68). Additionally, 310 MASLD patients diagnosed by imaging or biopsy were included after the exclusion criteria (Supplemental Figure S2). The clinical characteristics showed significant differences across the five groups (Table 1). Most of the parameters, except for BUN were significantly higher in the CC + SLD-, MASLD and MetALD compared to the CC-SLD-. Notably, parameters, except for BP, platelet and creatinine, were significantly higher in MASLD than in CC + SLD-. The incidence of liver dysfunction (ALT > 33 for men, ALT > 25 for women) was 7.1% in CC-SLD-, 18.5% in CC + SLD-, 53.1% in MASLD (general adults), 36.2% in MetALD, 11.8% in CC-SLD + , and 79.4% in MASLD (patients). HSI and FLI were significantly elevated in MASLD and

Table 1 Characteristics of the enrolled general adults and patients

	General adults					Patients
	CC-SLD- (N = 1756)	CC + SLD- (N = 8900)	MASLD (N = 890)	MetALD (N = 262)	CC-SLD + (N = 262)	MASLD (N = 310)
Sex (M/F)	698/1058	361/529	361/ 529	225/ 37*†	32/ 36	95/215‡
Age	60 ± 10	60 ± 10	60 ± 10	60 ± 10	59 ± 12	59 ± 15‡
BMI	20 ± 2*	26 ± 4*†	26 ± 4*†	26 ± 3*†	21 ± 1*	27 ± 5‡
Waist circumference (cm)	75 ± 6*	91 ± 9*†	91 ± 9*†	91 ± 8*†	79 ± 5*†	NA
BP (mmHg)						
Systolic	120 ± 14*	130 ± 15†	130 ± 15†	133 ± 14†	121 ± 16*	NA
Diastolic	72 ± 8*	79 ± 10†	79 ± 10†	82 ± 10*†	73 ± 10*	NA
Hemoglobin (g/dL)	13.8 ± 1.3*	14.2 ± 1.4*†	14.2 ± 1.4*†	15 ± 1.2*†	14.1 ± 1.3	13.9 ± 1.8
Platelet (10*4/μL)	23 ± 5*	24 ± 6†	24 ± 6†	22 ± 5*	22 ± 5*	21 ± 11‡
FBG (mg/dL)	84 ± 7*	95 ± 21*†	95 ± 21*†	101 ± 27*†	86 ± 7*	116 ± 32‡
HbA1c (%)	5.3 ± 0.2*	5.8 ± 0.6*†	5.8 ± 0.6*†	5.7 ± 0.7†	5.3 ± 0.2*	6.2 ± 0.9‡
TG (mg/dL)	79 ± 27*	144 ± 84*†	144 ± 84*†	165 ± 122*†	83 ± 25*	147 ± 134
HDL cho (mg/dL)	70 ± 16*	55 ± 13*†	55 ± 13*†	56 ± 13*†	65 ± 14†	51 ± 15‡
LDL cho (mg/dL)	124 ± 28*	126 ± 32	126 ± 32	117 ± 31*†	124 ± 26	115 ± 32‡
AST (IU/L)	23 ± 9*	29 ± 15*†	29 ± 15*†	30 ± 17*†	23 ± 5	56 ± 49‡
ALT (IU/L)	18 ± 9*	35 ± 25*†	35 ± 25*†	33 ± 21*†	21 ± 8	70 ± 58‡
GGTP (IU/L)	26 ± 37*	43 ± 41*†	43 ± 41*†	74 ± 86*†	34 ± 33	74 ± 118‡
UA (mg/dL)	4.9 ± 1.2*	5.5 ± 1.2*†	5.5 ± 1.2*†	6.1 ± 1.4*†	4.9 ± 1.3	6 ± 1.5‡
BUN (mg/dL)	15 ± 4	15 ± 4	15 ± 4	15 ± 4	14 ± 3	14 ± 5
Creatinin (mg/dL)	0.7 ± 0.23*	0.71 ± 0.18	0.71 ± 0.18	0.79 ± 0.16*†	0.72 ± 0.15	0.73 ± 0.22
HSI	28 ± 2*	37 ± 6*†	37 ± 6*†	35 ± 5*†	30 ± 3*†	40 ± 7‡
FLI	9 ± 7*	46 ± 25*†	46 ± 25*†	53 ± 24*†	14 ± 11*	NA
FIB-4	1.54 ± 0.65*	1.35 ± 0.63†	1.35 ± 0.63†	1.58 ± 0.80*	1.45 ± 0.62	2.46 ± 1.78‡

As a result of matching for sex and age among the three groups (CC-SLD, CC + SLD, and MASLD), the final dataset included 11,876 general adults and 310 patients

Values are mean ± SD

CC-SLD- Cardiometabolic criteria negative and steatotic liver disease negative, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative, *MASLD* metabolic dysfunction-associated steatotic liver disease, *MetALD* metabolic dysfunction and alcohol-associated liver disease, *CC-SLD +* Cardiometabolic criteria negative and steatotic liver disease positive, *BMI* body mass index (weight (kg) / height^2 (m^2)), *BP* blood pressure, *FBG* fasting blood glucose, *TG* triglyceride, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGTP* gamma-glutamyl transpeptidase, *UA* uric acid, *HSI* hepatic steatosis index, *FLI* fatty liver index, *FIB-4* fibrosis 4 index

* < 0.05 vs. CC + SLD-

† < 0.05 vs. CC-SLD-

‡ < 0.05 MASLD (General adults vs patients)

MetALD compared to CC + SLD-. Fibrosis-4 index (FIB-4) (Vallet-Pichard et al. 2007) was significantly higher in patients with MASLD compared to the general adults with MASLD. Furthermore, most of these parameters were worse in patients with MASLD compared to general adults with MASLD. The matching rates of OBE, GLC and TG in individuals meeting the CC were significantly higher in the MASLD and MetALD compared to CC + SLD- (Supplemental Figure S3). The matching rate of HT was significantly higher in MetALD compared to the other groups. These results indicate that the presence of steatosis with CC corresponds to a more aggravated metabolic status than in cases of CC positive without steatosis.

The imbalances of FAAs observed in SLD and CC

We investigated the profiles of FAAs among five groups (Table 2). In MASLD, out of 20 FAAs, 15 FAAs; glutamic acid (Glu), proline (Pro), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), cystine (Cys), Met, Tyr, phenylalanine (Phe), ornithine (Orn), histidine (His), Lysine (Lys), tryptophan (Trp), and arginine (Arg) were significantly increased compared to CC-SLD-. Additionally, 12 FAAs; Glu, Pro, Ala, Val, Leu, Ile, Cys, Met. Tyr, Phe, Orn and Trp showed a significant increase in MASLD compared to CC + SLD-. Gly and asparagine (Asn) were significantly decreased, while Thr and Ser showed no change in MASLD compared to both CC-SLD- and CC + SLD- (Table 2). Similar changes were more pronounced in MetALD compared to MASLD (Fig. 1A). In CC-SLD +, Val and Leu were significantly increased compared to CC-SLD-.

Cardiometabolic criteria associated with the imbalance of FAAs

We confirmed that 3,948 out of 8,900 subjects with CC + SLD- met only one of the five CC. We categorized the 3,948 subjects into five groups: OBE (n = 2249), GLC (n = 859), HT (n = 482), TG (n = 214) and HDL (n = 144) and investigated which CC were associated with change in

Table 2 The imbalances of FAAs observed in SLD and CC

AAs (nmol/mL)		CC-SLD- (N = 1756)	CC + SLD- (N = 8900)	MASLD (N = 890)	MetALD (N = 262)	CC-SLD + (N = 68)
Threonine	(Thr)	183 ± 38*	187 ± 40†	185 ± 40‡	192 ± 42†	186 ± 45
Serine	(Ser)	93 ± 14	93 ± 15	93 ± 16	91 ± 17	93 ± 15
Asparagine	(Asn)	56 ± 11	56 ± 12	54 ± 11*†‡	56 ± 11	54 ± 11
Glutamic acid	(Glu)	46 ± 12*	56 ± 16†	63 ± 17*†‡	68 ± 18*†	50 ± 14*
Glutamine	(Gln)	504 ± 60*	497 ± 63†	497 ± 62†‡	466 ± 65*†	501 ± 66
Proline	(Pro)	155 ± 40*	166 ± 42†	173 ± 44*†‡	183 ± 37*†	160 ± 40
Glycine	(Gly)	225 ± 57*	213 ± 57†	200 ± 47*†‡	186 ± 39*†	220 ± 54
Alanine	(Ala)	308 ± 70*	342 ± 76†	373 ± 76*†	379 ± 68*†	330 ± 81
Valine	(Val)	194 ± 35*	212 ± 39†	229 ± 39*†	232 ± 37*†	207 ± 36†
Leucine	(Leu)	109 ± 21*	118 ± 24†	125 ± 23*†‡	135 ± 22*†	117 ± 23†
Isoleucine	(Ile)	55 ± 13*	61 ± 16†	66 ± 17*†‡	69 ± 15*†	59 ± 14
Cystine	(Cys)	57 ± 13*	59 ± 14†	62 ± 16*†‡	68 ± 16*†	55 ± 15*
Methionine	(Met)	26 ± 4*	27 ± 4†	28 ± 4*†‡	28 ± 4*†	26 ± 4
Tyrosine	(Tyr)	63 ± 13*	68 ± 14†	73 ± 15*†‡	76 ± 15*†	65 ± 13
Phenylalanine	(Phe)	59 ± 10*	62 ± 10†	64 ± 10*†	65 ± 10*†	61 ± 9
Ornithine	(Orn)	68 ± 15*	69 ± 14†	70 ± 15†	70 ± 13	66 ± 13
Histidine	(His)	83 ± 10*	85 ± 11†	85 ± 11†‡	89 ± 12*†	85 ± 12
Lysine	(Lys)	129 ± 21*	133 ± 21†	136 ± 20*†‡	139 ± 18*†	129 ± 22
Tryptophan	(Trp)	51 ± 9*	52 ± 10†	55 ± 9*†‡	56 ± 10*†	51 ± 10
Arginine	(Arg)	49 ± 11*	51 ± 11†	52 ± 12†	53 ± 11†	50 ± 11
Total 20 AAs		2330 ± 252*	2419 ± 270†	2498 ± 264*†	2509 ± 224*†	2380 ± 265

Values are mean ± SD

AAs amino acids, *CC-SLD-* Cardiometabolic criteria negative and steatotic liver disease negative, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative, *MASLD* metabolic dysfunction-associated steatotic liver disease, *MetALD* metabolic dysfunction and alcohol-associated liver disease, *CC-SLD +* Cardiometabolic criteria negative and steatotic liver disease positive

* < 0.05 vs. CC + SLD-

† < 0.05 vs. CC-SLD-

‡ < 0.05 MASLD vs MetALD

FAAs. Hyperaminoacidemia was observed in the GLC, TG and the HDL, and changes in FAAs were similar among these groups (Table 3). Notably, in the GLC, all FAAs except for Gly were significantly increased compared to CC-SLD-. In the OBE and HT, the changes in FAAs were subtle and Ser, Asn, Gln, and Gly were significantly decreased in the OBE compared to CC-SLD-. FAAs changes were more pronounced with higher CC scores, with most FAAs increasing, especially in the presence of steatosis (Table 4). Similar trends were observed in MASLD patients as indicated by IR (Supplemental Table S3). Interestingly, while Thr levels were increased with CC score in CC + SLD-, this trend was not observed in MASLD. Moreover, in subject with CC score 4 and 5, the level was significantly lower in MASLD compared to CC + SLD- (Table 4). The levels of Gly and Gln gradually decreased with higher CC scores, regardless of steatosis, while the levels of Ser and Asn were unaffected by the CC score. Regarding the association between BW change and FAA levels (Table 5), a BW change of 2 percent did not significantly affect FAA levels except Cys in CC-SLD-. However, the BW change significantly changed the levels of some FAAs in CC + SLD- or MASLD. These changes were consistent with those observed by CC score (Table 4). In contrast, Ser and Gly levels significantly decreased in MASLD. Taken together, these findings suggest that a greater number of metabolic abnormalities are associated with increased levels of most FAAs, highlighting the underlying role of IR. However, some FAAs, particularly Gly, Ser and Thr, showed different change by steatosis.

The change of FAAs by progression in patients with MASH

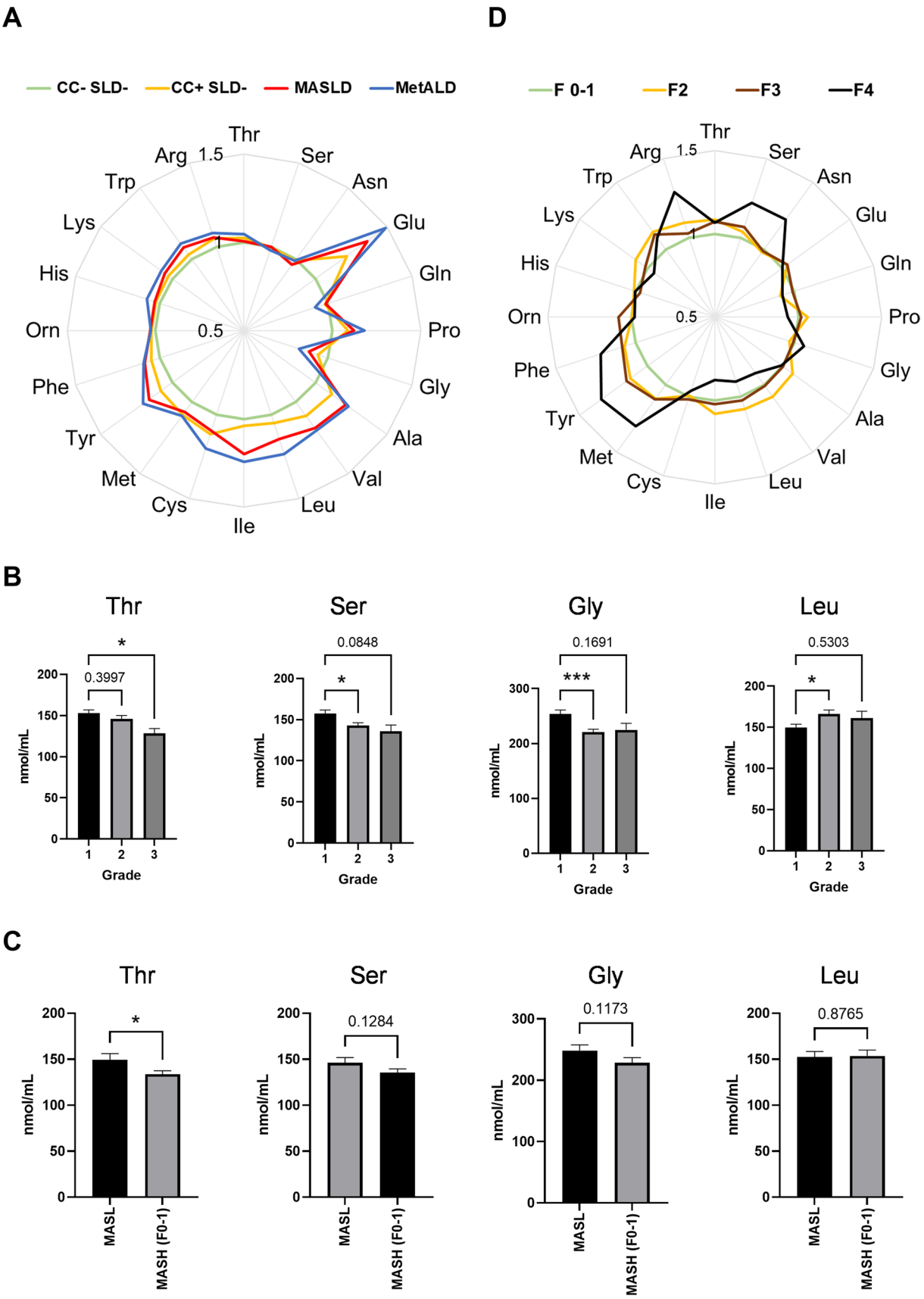
Next, we analyzed the changes in FAAs according to the pathological feature of the liver in patients with MASLD. As expected, The levels of Thr, Ser and Gly were significantly decreased in MASLD patients with steatosis grade 2 or 3 (Fig. 1B, Supplemental Table S4). In contrast, Leu significantly increased in patients with steatosis grade 2. Gly decreased according to inflammation grade as well as steatosis grade, while Tyr increased according to Ballooning grade (Supplemental Table S4). Consistent with these data, Thr was significantly decreased in early MASH (F0 or F1) compared to MASL. Ser and Gly also tended to decrease (Fig. 1C). Regarding fibrosis, the levels of Ser, Asn, Met, Tyr and Phe were significantly increased in F4 cirrhosis. BCAAs, especially Val, tended to increase ($p = 0.09$) at F2 and then decreased after F2. Accompanying these changes, Fischer's ratio gradually decreased according to fibrosis stage (Fig. 1D, Supplemental Table S5).

The change of FAAs in liver of MASLD model mice

To explore the imbalance of FAAs and the energy metabolism in the steatotic liver, we conducted metabolomics on the liver of MASLD model mice. We confirmed that a 25-week FFC diet (Supplemental Figure S4A) induced MASLD phenotypes, including obesity, liver dysfunction, hyperglycemia, and hyperlipidemia in middle-aged mice (50-week-old) as shown in Fig. 2A. Consistent with these data, liver and adipose tissue weights increased, but muscle weight was unchanged compared to the normal diet (Supplemental Figure S4B, S4C). Pathologically, steatosis and fibrosis were observed in the liver (Fig. 2B). Electron microscopy revealed that the FFC diet led to mitochondrial dysfunction and endoplasmic reticulum (ER) stress in steatotic hepatocytes, indicated by mitochondrial round formation and ER dilation (Fig. 2B). Most of the FAAs, except for Cys and Arg, were decreased in the liver due to the FFC diet, regardless of mouse age (Supplemental Table S6). Specifically, Gly, Ser, Asn, and Tyr significantly decreased after just 2 weeks on the diet. CE-TOFMS detected 98 metabolites, including FAAs, in the liver and revealed distinct metabolic profiles between the NC and the FFC diet through principal component analysis (Supplemental Figure S4D). Pathway enrichment analysis between the NC and the FFC diet showed that Ser, Gly and Thr metabolism was highly impacted among metabolic pathways (Fig. 2C). Consistent with these data, mitochondrial dysfunction was observed in the liver fed an FFC diet, as evidenced by significant decreases in ATP, ADP, and NAD + , as well as lactate and pyruvate, which are conversion products of Ser, Gly, and Thr (Fig. 2D). RNA-seq indicated that the metabolism of these FAAs was suppressed, and glycolysis was enhanced (Fig. 2E), while the expression of representative transporters for Ser and Thr was increased in the liver of mice fed an FFC diet. Summarized Gly-Ser-Thr metabolism was shown in Fig. 2F. Overall, these results indicate that the abnormal metabolism of Gly, Ser and Thr is strongly associated with the pathogenesis of MASLD in a mouse model.

Dietary intervention by Gly, Ser and Tyr ameliorated the pathogenesis of MASLD model mice

To explore the efficacy of dietary intervention by Gly, Ser and Thr, we compared the phenotypes of mice fed the control diet (AIN-93G) and the H-GST diet which has high Gly, Ser and Thr (Supplemental Table S2). In this experiment, the leptin deficient mouse, the ob/ob mouse, was employed (Supplemental Figure S5A) because a previous study elucidated that BCAAs were increased and Gly, Ser and Thr were decreased in plasma (She et al. 2007), which was similar to the FAAs profile in human. Interestingly, liver weight and size were significantly decreased in H-GST diet compared


A
CC- SLD-
CC+ SLD-
MASLD
MetALD
Thr
Ser
Asn
Glu
Gln
Pro
Gly
Ala
Val
Leu
Ile
Cys
Met
Tyr
Phe
Orn
His
Lys
Trp
Arg
1.5
1
0.5
D
F 0-1
F2
F3
F4
B
Thr
Ser
Gly
Leu
nmol/mL
Grade
0.3997
0.0848
0.1691
0.5303
C
MASL
MASH (F0-1)
0.1284
0.1173
0.8765

◂**Fig. 1** FAAs profile in peripheral blood of MASLD and the changes according to pathological features. **A** A radar chart of 20 plasma FAAs in general adults was presented. The axis indicates fold change relative to CC-SLD-. The light green, yellow, red, and blue lines represent CC-SLD-, CC + SLD-, MASLD, and MetALD, respectively. **B** The concentrations of four FAAs were compared according to steatosis grade in patients diagnosed by biopsy (n = 170) and p values were presented by steatosis grade. *p < 0.05, ***p < 0.001, vs. grade 1. **C** The concentrations of 4 FAAs were compared between MASLD and early MASH (Fibrosis stage 0 or 1). MASH was defined as patients with a fibrosis stage ≥ 2 or a NAS ≥ 4, and all others were defined as MASL. MASL; n = 27, early MASH; n = 30. The p values were presented. *p < 0.05. **D** A radar chart of 20 plasma FAAs was presented according to fibrosis stages in MASLD. The axis indicates fold change relative to F 0–1 (no fibrosis and F1). The light green, yellow, brown, and black lines represent F 0–1, F2, F3, and F4 (cirrhosis), respectively. *FAAs* free amino acids, *CC-SLD-* Cardiometabolic criteria negative and steatotic liver disease negative, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative, *MASLD* metabolic dysfunction-associated steatotic liver disease, *MetALD* metabolic dysfunction and alcohol-associated liver disease, *MASL* metabolic dysfunction-associated steatotic liver, *MASH* metabolic dysfunction-associated steatohepatitis

to the control diet although body weight was not changed (Fig. 3A, B). As expected, the H-GST diet improved the MASLD phenotype compared to the control diet both biochemically (Fig. 3C) and pathologically (Fig. 3D). The weights of adipose tissues and skeletal muscles were also not different between these diets (Supplemental Figure S5B). Fasting blood glucose (FBG), TG, and T-cho levels were also not different between diets (Supplemental Figure S5C). In the liver of ob/ob mice, the levels of FAAs were significantly decreased compared to wild-type mice (Fig. 3E), similar to the FFC diet (Supplemental Table S6). While the H-GST diet significantly increased the levels of Gly and Ser in the liver compared to the control diet, Thr showed the same trend. Interestingly, AAs which were low content in the H-GST diet were not decreased in the liver of mice fed H-GST diet. These results suggest that dietary interventions targeting Gly, Ser, and Thr could ameliorate the pathology of MASLD by restoring the levels of these AAs in the liver.

Table 3 The imbalances of FAAs caused by both SLD and each distinct cardiometabolic criterion

AAs	CC-SLD-	CC-SLD +	Cardiometabolic criteria				
			OBE	GLC	HT	TG	HDL
(nmol/mL)	(N = 1756)	(N = 68)	(N = 2249)	(N = 859)	(N = 482)	(N = 214)	(N = 144)
Thr	183 ± 38	186 ± 45	183 ± 38	188 ± 40*	186 ± 39	195 ± 38*	195 ± 41*
Ser	93 ± 14	93 ± 15	92 ± 14*	97 ± 16*	94 ± 15	92 ± 14	96 ± 16*
Asn	56 ± 11	54 ± 11	55 ± 11*	59 ± 13*	55 ± 10*	58 ± 11	59 ± 11*
Glu	46 ± 12	50 ± 14	51 ± 13*	49 ± 13*	47 ± 12*	58 ± 13*	46 ± 11
Gln	504 ± 60	501 ± 66	498 ± 60*	515 ± 60*	496 ± 62*	487 ± 60*	521 ± 62*
Pro	155 ± 40	160 ± 40	156 ± 37	160 ± 42*	155 ± 35	184 ± 37*	166 ± 37*
Gly	225 ± 57	220 ± 54	219 ± 60*	226 ± 60	219 ± 52*	219 ± 60	221 ± 43
Ala	308 ± 70	330 ± 81	317 ± 68*	333 ± 77*	309 ± 68	365 ± 68*	321 ± 68*
Val	194 ± 35	207 ± 36*	201 ± 35*	201 ± 38*	193 ± 33	213 ± 35*	208 ± 35*
Leu	109 ± 21	117 ± 23*	112 ± 21*	111 ± 22*	109 ± 20	120 ± 21*	120 ± 20*
Ile	55 ± 13	59 ± 14	57 ± 14*	58 ± 16*	55 ± 12	64 ± 14*	61 ± 13*
Cys	57 ± 13	55 ± 15	57 ± 13	59 ± 13*	56 ± 12	58 ± 13	56 ± 13
Met	26 ± 4	26 ± 4	26 ± 4	27 ± 4*	25 ± 4*	28 ± 4*	28 ± 3*
Tyr	63 ± 13	65 ± 13	65 ± 13*	66 ± 14*	63 ± 12	68 ± 13*	63 ± 10
Phe	59 ± 10	61 ± 9	59 ± 9	62 ± 11*	58 ± 9*	61 ± 9*	60 ± 8
Orn	68 ± 15	66 ± 13	67 ± 14*	71 ± 15*	66 ± 14*	72 ± 14*	71 ± 13*
His	83 ± 10	85 ± 12	84 ± 10*	84 ± 11*	83 ± 10	86 ± 10*	87 ± 11*
Lys	129 ± 21	129 ± 22	129 ± 20	134 ± 23*	126 ± 18*	132 ± 20	138 ± 22*
Trp	51 ± 9	51 ± 10	50 ± 9	52 ± 10*	50 ± 9	56 ± 9*	54 ± 9*
Arg	49 ± 11	50 ± 11	49 ± 11	52 ± 12*	50 ± 11*	51 ± 11*	51 ± 10*
Total 20 AAs	2330 ± 252	2380 ± 265	2344 ± 245	2415 ± 287*	2307 ± 240	2472 ± 245*	2425 ± 256*

OBE, GLC, HT, TG and HDL were each criterion in cardiometabolic criteria (Supplemental Table S1)

AAs amino acids, *CC-SLD-* Cardiometabolic criteria negative and steatotic liver disease negative, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative

* < 0.05 vs. CC + SLD-

Table 4 The difference of free amino acids levels between subjects with or without steatosis according to cardiometabolic criteria score

AAs	CC-SLD-	CC score (CC + SLD-)				CC-SLD +	CC score (MASLD)			
		1	2	3	4 and 5		1	2	3	4 and 5
(nmol/mL)	(N = 1756)	(N = 3948)	(N = 3134)	(N = 1401)	(N = 417)	(N = 68)	(N = 242)	(N = 345)	(N = 221)	(N = 82)
Thr	183 ± 38	186 ± 39	188 ± 40*	187 ± 41*	191 ± 40*	186 ± 45	185 ± 39	186 ± 39	182 ± 40	181 ± 45
Ser	93 ± 14	93 ± 15	93 ± 15	92 ± 16	92 ± 15	93 ± 15	93 ± 17	92 ± 16	92 ± 15	93 ± 16
Asn	56 ± 11	56 ± 12	56 ± 11*	55 ± 11*	56 ± 11	54 ± 11*	55 ± 13	54 ± 10*†	54 ± 12*	54 ± 11
Glu	46 ± 12	50 ± 14*	56 ± 15*	64 ± 17*	71 ± 19*	50 ± 14*	55 ± 15*†	61 ± 16*†	68 ± 17*†	74 ± 17
Gln	504 ± 60	502 ± 62	499 ± 63*	486 ± 65*	476 ± 59*	501 ± 66	504 ± 59	500 ± 63	491 ± 62*	481 ± 59
Pro	155 ± 40	159 ± 40*	167 ± 40*	180 ± 44*	190 ± 43*	160 ± 40	164 ± 41*†	173 ± 44*†	180 ± 42*	187 ± 46
Gly	225 ± 57	221 ± 59*	210 ± 55*	201 ± 54*	192 ± 43*	220 ± 54	207 ± 47*†	199 ± 47*†	199 ± 49*	183 ± 32
Ala	308 ± 70	322 ± 72*	345 ± 74*	373 ± 74*	400 ± 71*	330 ± 81*	344 ± 68*†	371 ± 73*†	395 ± 76*†	411 ± 77
Val	194 ± 35	201 ± 36*	214 ± 37*	228 ± 41*	243 ± 40*	207 ± 36*	213 ± 35*†	228 ± 36*†	237 ± 40*†	256 ± 41
Leu	109 ± 21	112 ± 21*	119 ± 23*	127 ± 26*	136 ± 26*	117 ± 23*	117 ± 20*†	124 ± 21*†	128 ± 24*	141 ± 24
Ile	55 ± 13	57 ± 14*	62 ± 16*	67 ± 18*	74 ± 18*	59 ± 14*	60 ± 15*†	66 ± 15*†	70 ± 18*†	77 ± 17
Cys	57 ± 13	57 ± 13	60 ± 14*	62 ± 15*	64 ± 16*	55 ± 15	58 ± 14	63 ± 16*†	64 ± 15*	67 ± 17
Met	26 ± 4	26 ± 4*	27 ± 4*	28 ± 4*	28 ± 4*	26 ± 4	27 ± 4*†	27 ± 4*†	28 ± 4*	29 ± 4
Tyr	63 ± 13	65 ± 13*	69 ± 14*	72 ± 15*	76 ± 16*	65 ± 13	69 ± 13*†	73 ± 15*†	76 ± 14*†	80 ± 15
Phe	59 ± 10	60 ± 10*	63 ± 11*	64 ± 11*	66 ± 11*	61 ± 9	62 ± 10*†	64 ± 10*†	66 ± 11*†	68 ± 10
Orn	68 ± 15	68 ± 14	70 ± 15*	71 ± 15*	73 ± 14*	66 ± 13	68 ± 15	70 ± 14	71 ± 16*	73 ± 14
His	83 ± 10	84 ± 10*	85 ± 11*	86 ± 11*	87 ± 11*	85 ± 12	84 ± 11	86 ± 10*	85 ± 11*	87 ± 12
Lys	129 ± 21	130 ± 20	134 ± 21*	135 ± 21*	139 ± 21*	129 ± 22	134 ± 19*†	136 ± 20*†	137 ± 19*	140 ± 22
Trp	51 ± 9	51 ± 9	53 ± 9*	54 ± 10*	56 ± 10*	51 ± 10	53 ± 9*†	55 ± 9*†	55 ± 10*	58 ± 10
Arg	49 ± 11	50 ± 11*	52 ± 11*	53 ± 11*	56 ± 12*	50 ± 11	51 ± 13*	51 ± 12*	52 ± 11*	52 ± 11
Total 20 AAs	2330 ± 252	2365 ± 261*	2432 ± 261*	2499 ± 272*	2575 ± 261*	2380 ± 265	2420 ± 253*†	2493 ± 252*†	2549 ± 266*†	2612 ± 270

AAs amino acids, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative, *CC-SLD-* Cardiometabolic criteria negative and steatotic liver disease negative, *CC score* the number of matches with Cardiometabolic criteria, *MASLD* metabolic dysfunction-associated steatotic liver disease

* < 0.05 vs. CC-SLD-

† < 0.05 vs. SLD- with the same CC score

Table 5 The levels of free amino acids by the change of body weight for a year

AAs	CC-SLD-			CC + SLD-			MASLD		
	> 2% loss	NC	> 2% gain	> 2% loss	NC	> 2% gain	> 2% loss	NC	> 2% gain
(nmol/mL)	(N = 737)	(N = 762)	(N = 217)	(N = 2691)	(N = 3969)	(N = 1989)	(N = 284)	(N = 400)	(N = 189)
Thr	182 ± 38	184 ± 38	185 ± 39	186 ± 40	187 ± 40	187 ± 39	185 ± 40	185 ± 41	183 ± 41
Ser	93 ± 14	92 ± 14	93 ± 15	94 ± 15*	92 ± 15	92 ± 16	93 ± 17	93 ± 16	90 ± 15*
Asn	56 ± 11	56 ± 11	57 ± 11	56 ± 11	56 ± 11	55 ± 12	56 ± 12*	54 ± 11	53 ± 11
Glu	46 ± 12	46 ± 12	45 ± 12	54 ± 16*	56 ± 16	57 ± 17*	60 ± 16	62 ± 16	67 ± 19*
Gln	504 ± 60	505 ± 60	507 ± 62	500 ± 63*	497 ± 62	493 ± 64*	505 ± 59	501 ± 62	477 ± 63*
Pro	151 ± 35	156 ± 40	161 ± 50	163 ± 41*	166 ± 41	171 ± 43*	169 ± 45	175 ± 42	178 ± 43
Gly	226 ± 60	224 ± 56	227 ± 56	213 ± 55	212 ± 56	213 ± 58	203 ± 49	201 ± 47	192 ± 42*
Ala	303 ± 67	310 ± 70	318 ± 73	336 ± 75*	341 ± 75	351 ± 79*	366 ± 77	377 ± 76	379 ± 74
Val	193 ± 35	194 ± 33	195 ± 39	208 ± 38*	212 ± 38	216 ± 42	224 ± 39*	231 ± 38	233 ± 41
Leu	109 ± 21	109 ± 19	108 ± 22	116 ± 24*	118 ± 23	120 ± 25*	123 ± 22	126 ± 22	126 ± 24
Ile	55 ± 13	55 ± 13	56 ± 16	60 ± 16*	61 ± 16	63 ± 18*	65 ± 16	67 ± 17	67 ± 17
Cys	58 ± 14*	56 ± 12	56 ± 17	59 ± 14	60 ± 14	59 ± 15*	61 ± 15	64 ± 16	62 ± 16
Met	26 ± 4	26 ± 4	26 ± 4	27 ± 4	27 ± 4	27 ± 4	27 ± 4	28 ± 4	28 ± 4
Tyr	63 ± 12	63 ± 12	63 ± 13	67 ± 14*	68 ± 14	69 ± 15	72 ± 15	74 ± 14	74 ± 15
Phe	59 ± 10	59 ± 9	59 ± 11	61 ± 10*	62 ± 10	62 ± 10	64 ± 11	65 ± 10	64 ± 11
Orn	68 ± 15	68 ± 15	68 ± 15	69 ± 14	70 ± 14	69 ± 15	70 ± 15	70 ± 15	70 ± 15
His	82 ± 11	83 ± 10	83 ± 10	84 ± 11*	85 ± 11	86 ± 11	85 ± 10	86 ± 11	84 ± 11
Lys	130 ± 20	129 ± 20	128 ± 22	132 ± 21	133 ± 21	132 ± 21	137 ± 20	137 ± 19	133 ± 20*
Trp	51 ± 9	51 ± 9	51 ± 10	52 ± 10	52 ± 9	53 ± 9	55 ± 10	55 ± 9	54 ± 10
Arg	49 ± 10	49 ± 10	50 ± 12	52 ± 11	51 ± 11	51 ± 12	52 ± 12	52 ± 12	51 ± 12
Total 20 AAs	2324 ± 241	2331 ± 250	2350 ± 281	2403 ± 264*	2420 ± 266	2440 ± 281*	2486 ± 265	2516 ± 264	2482 ± 267

The three groups were classified based on a 2% body weight change over the past year, as assessed by a questionnaire, and various amino acids were compared among the groups

Data are presented as means ± SD. * < 0.05vs. NC

AAs amino acids, *CC-SLD-* Cardiometabolic criteria negative and steatotic liver disease negative, *CC + SLD-* Cardiometabolic criteria positive and steatotic liver disease negative, *MASLD* metabolic dysfunction-associated steatotic liver disease, *NC* not change

Discussion

In this study, we elucidated the profiles of FAAs in the peripheral blood of subjects with SLD. The principal findings of this study are: (i) The concentrations of 16 FAAs were significantly increased in accordance with cardiometabolic abnormalities accompany by IR but Ser, Asn, Gln and Gly were significantly decreased or remained unchanged; (ii) Out of the 16 FAAs, 12 increased further, but Thr, Orn, His and Arg remained unchanged in MASLD (general adults); (iii) Thr, Ser and Gly were significantly decreased in MASLD patients with steatosis grades 2 or 3; (iv) These 3 FAAs were significantly decreased in the liver of MASLD mice and associated with mitochondrial dysfunction and enhanced glycolysis; (v) Correction with these FAAs improved the pathogenesis of MASLD in mouse models. These data provide a metabolic link between steatosis and other metabolic abnormalities via FAAs. A previous systematic review and meta-analysis elucidated that high levels of BCAAs (Val, Leu and Ile), AAAs (Tyr, Phe) and Ala were associated with the risk of type 2 diabetes (Morze et al. 2022). We first elucidated that 16 FAAs including the 6 AAs were gradually increased according to CC, especially both hyperglycemia and hypertriglyceridemia based on IR, and we confirmed that total AAs and 5 AAs of the 6 AAs were significantly increased in MASLD patients with high homeostatic model assessment for IR (HOMA-IR) (Supplemental Table S3). These data suggest that "hyperaminoacidemia" also occurred based on IR. However, 4 FAAs, Ser, Gly, Asn, and Gln were not increased by IR. Interestingly, Thr was increased by CC score without steatosis, but this trend was not observed in MASLD. This result may indicate that the demand for Thr in liver increases due to steatosis.

We focused on the Thr, Ser and Gly, and performed mouse experiments because Gln and Asn are known to be unstable FAAs which easily convert to Glu and Asp by pH and enzyme in plasma, and their decrease was not observed in MASLD patients. The metabolism among Thr, Ser, Gly,

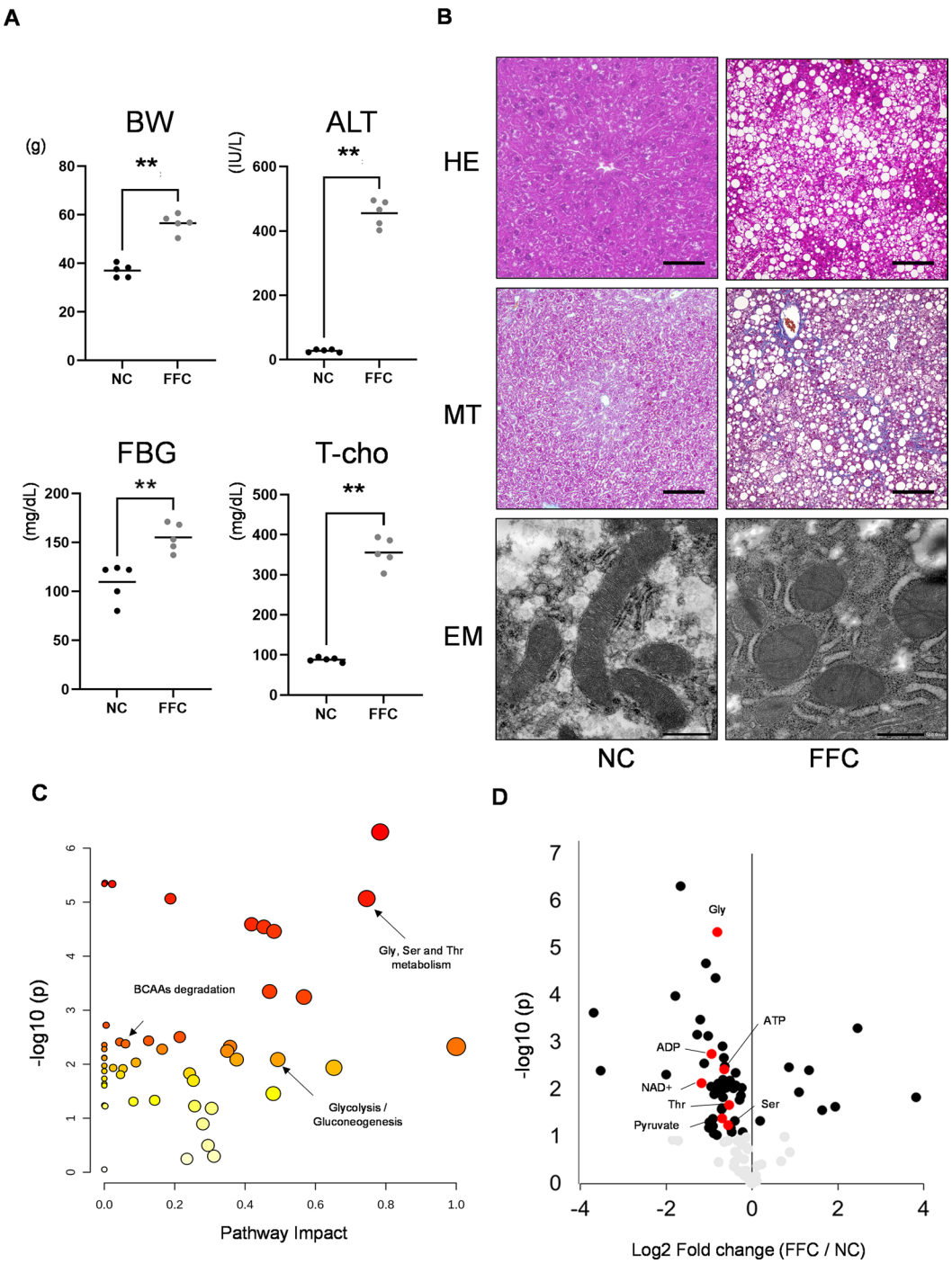


Fig. 2 The FFC diet induced an imbalance of FAAs and mitochondrial dysfunction in the mouse liver. **A** The comparison of MASLD phenotypes between normal chow (NC) diet and high-fat, fructose and cholesterol (FFC) diet. *BW* body weight, *ALT* alanine aminotransferase. *FBG* fasting blood glucose, *T-cho* total cholesterol. **B** Upper images were hematoxylin–eosin (HE) staining. Middle images were Masson's trichrome (MT) staining. Scale bar: 200 µm. Bottom images were electron microscopy (EM) images. Scale bar: 500 nm. **C** Metabolic pathway analysis. Vertical axis: p value by logarithmic scale. Horizontal axis: pathway impact values were calculated from pathway topology analysis by MetaboAnalyst. The node color was based on its p value and the node size was determined based on their pathway impact values. Detailed information regarding the analysis is provided in the supplementary information. **D** Volcano plot of metabolite differences in liver between NC and FFC diet. Red dots indicate representative metabolites in Fig. 2F. Red and Black dots were significantly different ($p < 0.01$) between NC and FFC diet, but gray dots were not significant. **E** Volcano plot of RNA-seq in liver tissue between NC and FFC diet. Red dots represent the enzyme genes mediating the metabolism of Gly, Ser, and Thr. Blue dots indicate the enzyme genes mediating glycolysis. Green dots represent the genes of Ser and Thr transporters. **F** A metabolic pathway diagram integrating metabolome analysis and RNA-seq results. Vertical axis units of each graph are nmol/ g (liver tissue). * $p < 0.05$

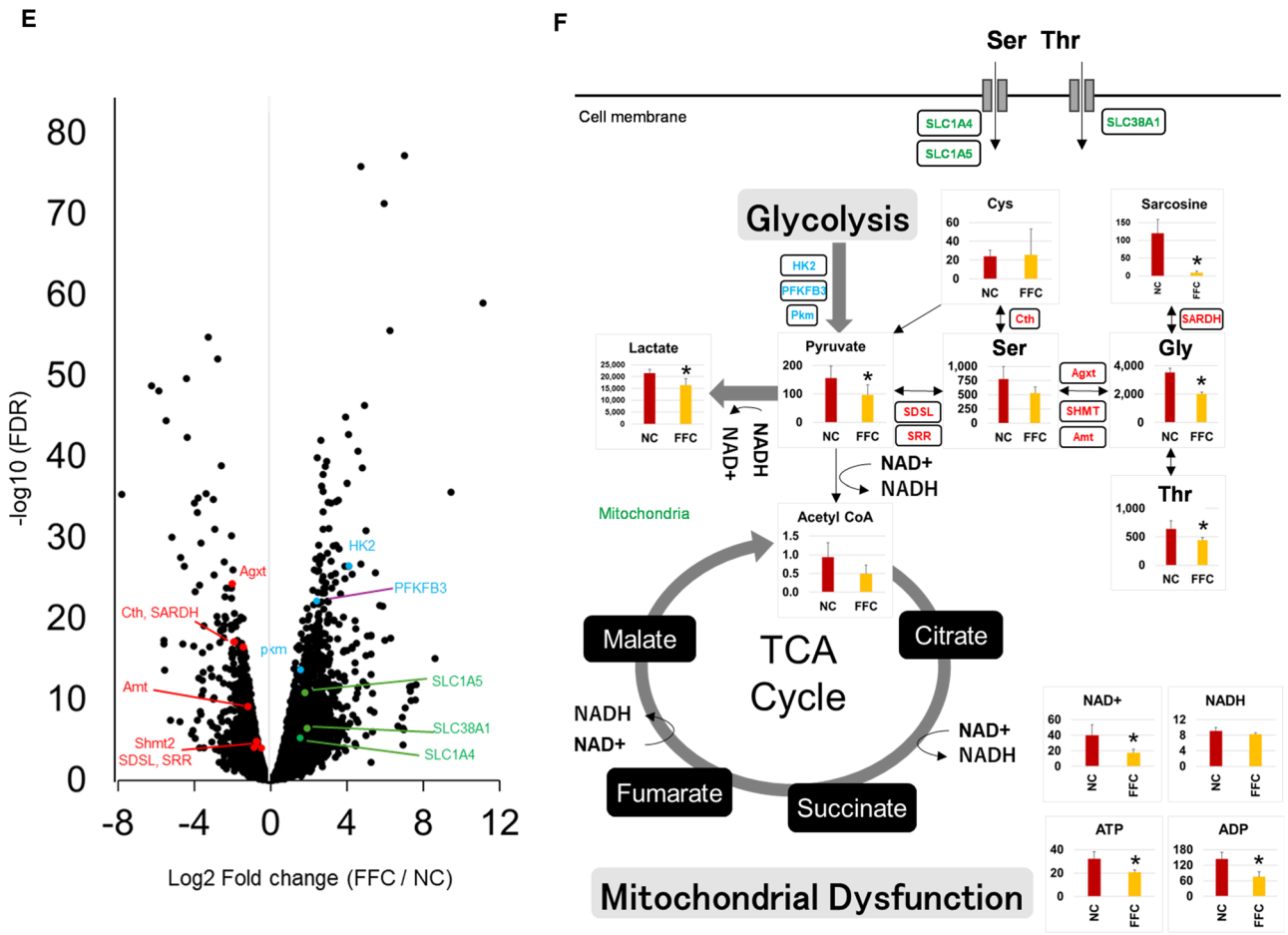


Fig. 2 (continued)

and glucose is known to be closely related (Pai et al. 2015; McBride et al. 2024). These FAAs metabolized to pyruvate as well as substrate of one-carbon metabolism (Ducker and Rabinowitz 2017). Serine hydroxy-methyltransferase mediates these metabolism (Malatesta et al. 2024). In this study, metabolomics and RNA sequencing analyses indicated that an FFC diet induced glycolysis and mitochondrial dysfunction, accompanied by a decrease in these three FAAs in liver. Furthermore, Ser and Gly were already decreased in liver before appearance of steatosis in mice fed FFC diet at 2 weeks. We previously reported that the concentrations of these FAAs were significantly lower in fasting hepatic portal blood of 6 weeks ob/ob mice with steatotic liver, even though glucose and free fatty acids were significantly higher than that of wild-type mice (Sano et al. 2021). These results suggest that insufficient levels of these FAAs aggravate mitochondrial dysfunction due to decreasing pyruvate or acetyl CoA. Alternatively, glycolysis was enhanced to keep ATP level. In this study, we have for the first time elucidated the efficacy of Ser and Thr as well as Gly in the pathogenesis of MASLD. Recent studies demonstrated that Gly administration ameliorated the pathogenesis of MASLD via glutathione synthesis or modulating innate immunity (Takashima et al. 2016; Rom et al. 2020; Ghrayeb et al. 2024). These studies support our data on the effect of Gly. However, the efficacy of Ser and Thr was not demonstrated in these studies because the Ser and Thr levels of plasma were higher in the Western diet model, which was quite different from that of human in this study. In contrast, the imbalance of plasma FAAs in ob/ob mice was similar to that of human because Ser and Thr levels in plasma were not increased (She et al. 2007). Taken together, our data suggest that administration of Gly, Ser, and Thr may convert hepatic anaerobic energy metabolism to aerobic energy metabolism by improving mitochondrial function mediated by pyruvate in MASLD, regardless of IR. Conversely, for FAAs with elevated plasma concentrations due to IR, it appears challenging to ameliorate MASLD pathogenesis through their administration, even though their levels are decreased in the liver.

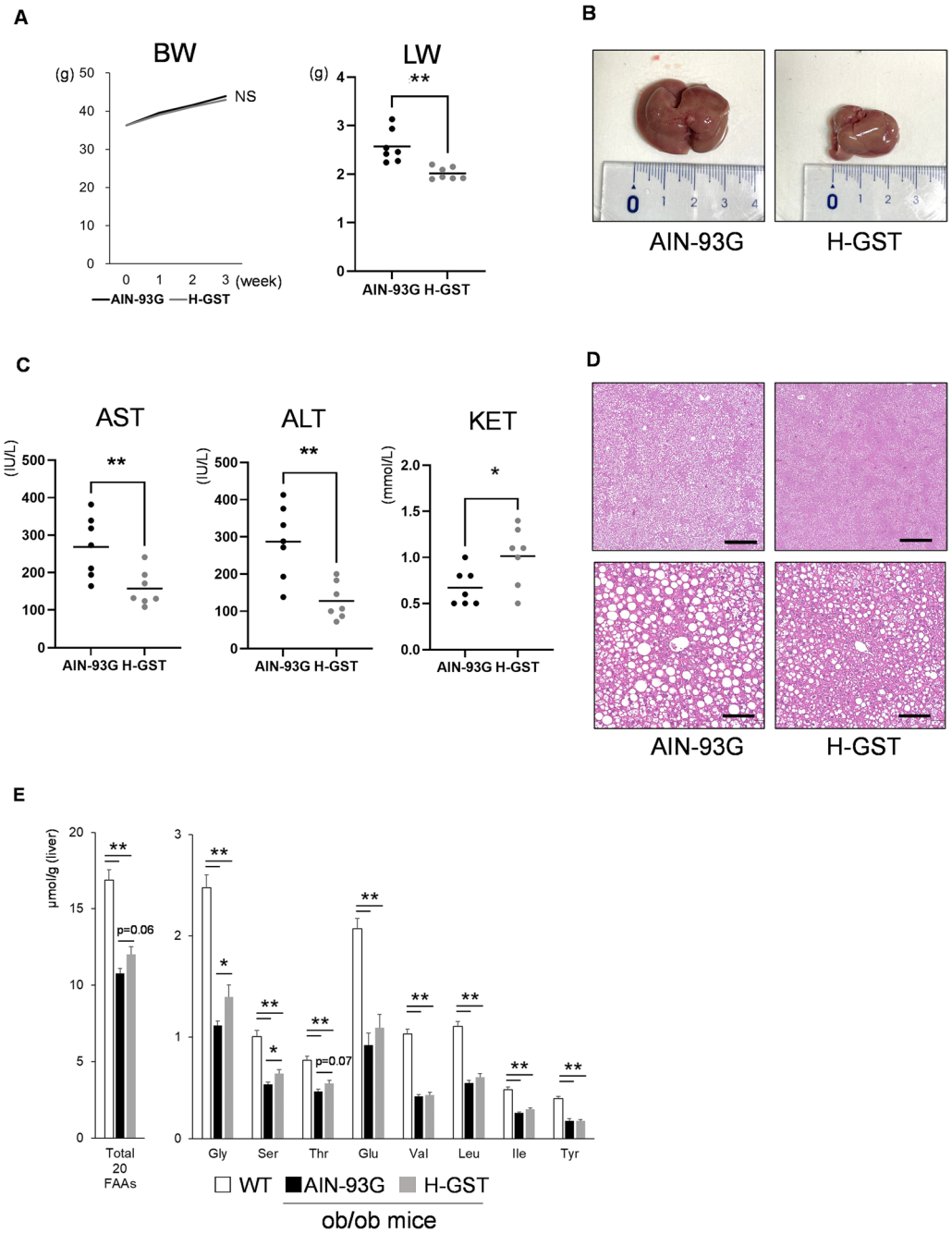


Fig. 3 The high Gly, Ser and Thr (H-GST) diet ameliorated the pathogenesis of MASLD in mice. **A** Left graph: Body weight (BW) gain of mice fed AIN-93G and H-GST. *NS* not significant. Right graph: Comparison of liver weight (LW) between each diet. **B** Macroscopic appearance of each liver. **C** Biochemical data of plasma in each fed mouse. KET: ketone body (**D**) Images were hematoxylin–eosin (HE) staining. Scale bar: 1 mm (upper images) and 200 μm (bottom images). **E** Comparison of the concentration of free amino acids (FAAs) in the liver among 9-weeks-old wild-type (WT) mice, ob/ob mice fed AIN-93G, and ob/ob mice fed H-GST diet for three weeks. **A**, **C**, **E** *$p < 0.05$, **$p < 0.01$

The strength of this study is that it is the first report elucidating human FAAs profiles in MASLD by analyzing a large number of the general population and patients. Then, using this FAAs profile, we found the AA candidates to improve the pathogenesis and elucidated the efficacy of Ser and Thr as well as Gly to MASLD model mice. However, there are some limitations. As the first limitation, we could not elucidate the detailed metabolic mechanisms involving these AAs. We need to investigate the kinetics of these AAs in organs at the cellular level using stable-isotope tracing

method in future study. Furthermore, this study was conducted using only male mice, and it is necessary to clarify the metabolic differences based on sex. As the second limitation, SLD in general adults is diagnosed through a questionnaire, not by imaging or biopsy. Therefore, the existence of SLD in groups without diagnosed SLD could not be ruled out, given the low prevalence of SLD (5.3%) in this study. However, all clinical characteristics, especially HSI and FLI, were reasonable between MASLD and CC + SLD-. As third limitation, we could not compare the difference of FAAs between general adults and patients with SLD because almost FAAs were higher in patients than general adults by inter-facility disparity even if quantitative data.

In summary, we elucidated the imbalance of FAAs observed in MASLD. Most of FAAs were increased in concordance with cardiometabolic parameters associated with IR. Interestingly, three FAAs (Gly, Thr and Ser) were significantly decreased with the steatosis grade in patients with MASLD. Their correction prevented the development of MASLD in model mice. This study provides a profile of FAAs in MASLD and suggests a future approach for nutritional therapy through FAA metabolism.

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Author contributions M.M. was responsible for the data acquisition, mouse experiments, analysis, interpretation of data and manuscript preparation; E.K. was responsible for the conception and design of the study, data acquisition, statistical analysis and interpretation of data, manuscript preparation, final drafting of the manuscript; A.S., M.T., K.K., T.K. and J.I. were responsible for the data acquisition and interpretation of data; H.M. contributed to the conception and design of the study; K.S., Y.A. and M.I. contributed to sample collection and reviewing the study; M.M., T.Y., T.M., S.Y., A.M. and T.K. contributed to the interpretation of data and critically reviewed the study for important intellectual content.

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Declarations

Conflicts of interest The authors declare no competing interests.

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