Supplementary Methods and Figures

Gut microbiota-derived metabolite isovalerylcarnitine modulates salt sensitivity of blood pressure and incident hypertension: a multicenter dietary salt intervention trial

Supplementary Methods

1. The Metabolome, Microbiome, and Dietary Salt Intervention (MetaSalt) study

1.1 Study design and study population

The MetaSalt study was a multicenter dietary salt intervention (non-randomized and self-controlled) trial conducted in four counties of northern China (Xinle, Nangong, Yuxian, and Changge) in 2019. The study was registered in the Chinese Clinical Trial Registry database on August 15, 2019 (registration number: ChiCTR1900025171). More detailed information about the MetaSalt study can be found in the published protocol[1]. Briefly, this study aims to identify the key gut microbes and metabolites that are influenced by the high-salt diet and identify the gut-microbial and metabolomics signatures of salt sensitivity (SS) of blood pressure (BP) (SSBP). Participants consisted of probands and their family members (including siblings, offspring, spouses, and parents). A proband was defined as the first person in a family whose systolic BP (SBP) was 130-159 mmHg and/or diastolic BP (DBP) was 85-99 mmHg. Probands' family members should meet with SBP < 160 mmHg and DBP < 100 mmHg. Other criteria included: 1) aged 18-60 years; 2) without taking antihypertensive medications or medications affecting BP and antibiotics within one month before screening; 3) with a negative urine protein test; 4) with a normal blood glucose level (fasting blood glucose < 7.0 mmol/L and postprandial blood glucose < 11.1 mmol/L). Before recruitment, the sample size was determined, and 456 participants would be appropriate to achieve 90% power for the detection of a 2.0 mmHg difference in SBP between intervention stages at a significance level of 1×10^{-5}. To account for a potential dropout rate of 10%, we aimed to recruit at least 500 participants. Ultimately, we recruited 528 participants from 361 families, including 359 probands and 169 family members. All included participants should consent to attend field centers for 23 consecutive days and adhere to complete the entire trial, including a 3-day baseline survey, a 10-day low-salt intervention, and a 10-day high-salt intervention. Of these participants, 512 (96.97%) completed the low-salt intervention and 503 (95.27%) finished the entire trial.

1.2 Data collection and measurements

Demographic characteristics, medical history, dietary factors, and lifestyle factors of each

participant in the MetaSalt study were collected by trained investigators using a standard questionnaire during the baseline survey. Participants who have smoked at least 100 cigarettes in the past were defined as smokers, and those who have drunk more than 12 times in the past year were defined as drinkers. Anthropometric measurements, including body weight, height, and waist circumference, were measured. Body mass index (BMI) was calculated as body weight divided by the square of height (kg/m^2). A simple standardized food frequency questionnaire was used to collect baseline dietary factors. Participants were asked to provide information about the consumption frequency of each food group over the past year. The baseline dietary score was calculated by referring to the Chinese guideline on healthy lifestyles to prevent cardiometabolic diseases[2].

1.3 Sample collection and biochemical measurement

On the first day of the baseline stage and the ninth day of the low-salt and high-salt intervention stages, fasting blood samples were collected from all participants. Plasma was immediately separated and shipped to the Beijing laboratory via cold chain for storage at -80 °C. The blood levels of fasting glucose, triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were measured. When TG < 400 mg/dL, low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation (LDL-C = TC − HDL-C − TG/5). Additionally, blood creatinine was measured to determine the estimated glomerular filtration rate (eGFR)[3]. Fecal samples of each participant were collected once during the baseline stage, the last two days of the low-salt period, and the last two days of the high-salt period, using a gut microbiome DNA collection kit (Genotek OMR-200, USA). After that, fecal samples were shipped to the Beijing laboratory via cold chain and then stored in -80 °C refrigerators. One 24-hour and two overnight urine samples were collected for each participant during the last three days of each study phase. Urinary samples were shipped to the Beijing laboratory via cold chain and then stored in -20 °C refrigerators. Furthermore, the urinary sodium (UNa) and urinary potassium (UK) of each urinary sample were measured. The mean levels of 24-hour UNa and 24-hour UK in each study phase were calculated. To obtain these data, the overnight UNa nd UK were converted to 24-hour values based on formulas developed from a random subsample of 238 GenSalt participants who had collected overnight and 24-hour urine on the same days at baseline and in each phase of the intervention[4,5].

Study phase	Formula
UNa, mmol	
Baseline	24 hour UNa = 111.62 + 1.77 × Overnight UNa
Low-salt intervention	24 hour UNa = 23.53 + 1.56 × Overnight UNa
High-salt intervention	24 hour UNa = 155.00 + 1.05 × Overnight UNa
UK, mmol	
Baseline	24 hour UK = 18.40 + 1.76 × Overnight UK
Low-salt intervention	24 hour UK = 16.67 + 1.49 × Overnight UK
High-salt intervention	24 hour UK = 20.38 + 1.44 × Overnight UK

1.4 Intervention

After a 2-day screening, eligible participants received a 23-day investigation, including a 3-day baseline survey (maintaining their usual diet), a 10-day low-salt intervention (3 g/d of salt or 51.3 mmol/d of sodium), and a 10-day high-salt intervention (18 g/d of salt or 307.8 mmol/d of sodium). To ensure participants' compliance, several measures were taken: 1) During the low-salt and high-salt intervention periods, professional dietitians determined identical recipes for participants based on the dietary habits of local residents, and study staff prepared meals without adding salt. Study supervisors then added pre-packed salt to the cooked food of each participant. 2) All participants were requested to finish their meals at specific canteens under the supervision of study supervisors. 3) During the low-salt intervention period, some salt-free condiments were allowed to be added to the food to improve the flavor. 4) We provided participants with enough food to ensure balanced nutrition. Thus, participants were not advised to eat other foods outside the trial. In addition, three urinary samples were collected for each participant on the last three days of each study phase to assess compliance.

1.5 Primary outcome measures

An electronic sphygmomanometer (Omron® HBP1300, Japan) was used to measure participants' BP three times on each day of the baseline stage, and on days 2, 8, 9, and 10 of the low-salt and high-salt stages. Participants' BP levels were measured in a fasting state in the morning of each day. Before each measurement, participants were requested to avoid alcohol, smoking, exercising, and drinking caffeine-containing beverages for at least 30 min. In addition, they should keep sitting for at least 5 min. The average SBP and DBP levels were calculated separately for the three-day baseline period, as well as the last three days in both the low-salt

and high-salt periods, to control the fluctuations in BP within a single day and avoid the carryover effect from the previous study phase. Mean arterial pressure (MAP) levels were calculated, i.e., MAP = 1/3 × SBP + 2/3 × DBP.

We used MAP to determine SSBP, since MAP combined the information of SBP and DBP, and it was a well-used index to measure SSBP[6]. To define salt-resistance (SR) and SS individuals, we calculated the changes in MAP when switching from the baseline (i.e., mean BP of the three-day baseline) to the low-salt intervention (i.e., mean BP of the last three days in the low-salt intervention) (ΔMAP_{L-B}), and the changes of MAP when switching from the low-salt intervention (i.e., mean BP of the last three days in the low-salt intervention) to the high-salt intervention (i.e., mean BP of the last three days in the high-salt intervention) (ΔMAP_{H-L}). All participants were categorized into three groups: extreme SS individuals ($\Delta MAP_{L-B} \leq$ -10 mmHg or $\Delta MAP_{H-L} \geq$ 10 mmHg), moderate SS individuals ($\Delta MAP_{L-B} \leq$ -5 mmHg and > -10 mmHg or $\Delta MAP_{H-L} \geq$ 5 mmHg and < 10 mmHg), and SR individuals (the remaining participants). We then calculated each single-day response of MAP, i.e., the changes in MAP from the baseline (mean BP of the three-day baseline) to the second, eighth, ninth, and tenth day in the low-salt intervention, and the changes in MAP from the low-salt intervention (mean BP of the last three days in the low-salt intervention) to the second, eighth, ninth, and tenth day in the high-salt intervention. Trajectories of MAP responses during the entire trial in all SSBP groups were illustrated by averaging the MAP responses at each time point.

2. The Genetic Epidemiology Network of Salt Sensitivity (GenSalt) study

The GenSalt study was a dietary salt intervention trial, registered with ClinicalTrials.gov, number NCT00721721. The study was conducted during 2003-2005, aiming at identifying susceptibility genes influencing individuals' BP responses to dietary sodium and potassium intake in human population. The detailed description was published before[4]. In brief, the GenSalt participants were recruited from six field centers in rural north China. Participants' inclusion and exclusion criteria were the same as those of the MetaSalt study. After screening, 3,153 participants were enrolled, 1,906 participants took part in the dietary interventions, and 1,879 (98.58%) participants completed the entire trial. The study design, data collection, biomedical measurement, sample collection, intervention, and primary outcome measurements of the GenSalt study were similar to those of the MetaSalt study. A 28-day trial was implemented for each eligible participant, including a 3-day baseline observation, a 7-day low-salt intervention (3 g/d of salt or 51.3 mmol/d of sodium), a 7-day high-salt intervention (18 g/d of salt or 307.8 mmol/d of sodium), and a 7-day high-salt and potassium-supplement

intervention (307.8 mmol/d of sodium and 60 mmol/d of potassium). Participants' BP levels were measured three times in a fasting state during the morning of each day on the baseline observation and days 2, 5, 6, and 7 of each intervention period. Overnight fasting blood samples were drawn on the first day of the baseline and the sixth day of each intervention period. Three urinary specimens (one 24-hour and two timed-overnight) were collected during each day of the baseline period and the last three days of each intervention period.

The definition of SR and SS individuals was the same as that of the MetaSalt study. To reduce cost and ensure power, we applied a 1:1 matching method to select participants for the replication of the findings identified based on the MetaSalt study. We took all 1,879 participants into account, and selected eligible pairs as many as possible, according to age (± 5 years), sex, field centers, and MAP (± 5 mmHg). Eventually, 113 extreme SS individuals and 113 SR individuals were kept. Plasma targeted metabolomics profiling was performed for these 226 participants using the same method as that applied in the MetaSalt study. Given that the fecal specimens of the GenSalt participants were not collected, metagenomic sequencing was not performed.

3. The prospective cohort

The cohort used to assess the associations of metabolites with the prevalence of hypertension, the incidence of hypertension, and the progression of BP status was derived from the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA) and China Multi-Center Collaborative Study of Cardiovascular Epidemiology 1998 [China-MUCA (1998)]. Details of these cohorts have been described and published elsewhere[7]. Briefly, 15,540 participants of the InterASIA cohort were recruited during 2000-2001, and 11,480 participants of the China-MUCA (1998) were recruited during 1998. These two cohorts had the first follow-up during 2007-2008, and were further followed up in 2012-2015 based on unified protocols.

BP information and the use of antihypertensive medications were collected during the morning of each visit. BP levels were measured three times for each participant who was in a fasting state after a 5-min quiet rest in a seated position. All participants were asked to avoid alcohol, smoking, exercising, and drinking caffeine-containing beverages for at least 30 min before measurement. The average SBP and DBP were calculated using three BP readings. Hypertension was defined as average SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and/or taking antihypertensive medication in the past two weeks. Ideal BP status was defined as SBP < 120 mmHg and DBP < 90 mmHg and without taking antihypertensive medications. Prehypertension was defined as SBP of 120-139 mmHg and/or DBP of 80-89 mmHg and

without taking antihypertensive medications. Normotension included ideal BP status and prehypertension. The progression of BP status was defined as BP status increasing ≥ 1 step, including from ideal BP status to prehypertension or hypertension, from prehypertension to hypertension, from stage-1 hypertension to stage-2 or stage-3 hypertension, and from stage-2 hypertension to stage-3 hypertension.

Given that the China-MUCA (1998) participants' blood samples were not collected during the 1998 visit, and to ensure the consistency of follow-up intervals, we used the 2007-2008 visit as the baseline, and randomly selected a population (n = 3,907) with available blood samples from these two cohorts to perform plasma targeted metabolomics profiling. We used all participants to assess the associations between metabolites and the prevalence of hypertension. After further excluding participants without BP information during the 2012-2015 visit (n = 1,181), 2,726 participants were kept to evaluate the associations between metabolites and the risk of BP status increasing ≥ 1 step. After further excluding participants with hypertension during the 2007-2008 visit (n = 1,404), 1,322 participants were kept to estimate the predictive value of metabolites on the incidence of prehypertension or hypertension (Supplementary Fig. 16).

3. Animal experiments

All rats were housed under specific-pathogen-free conditions in a 12-hour light/dark cycle with free access to food and water. All animal experiments were conducted following the recommendations of the Ministry of Science and Technology of China, and the protocol was approved by the Experimental Animal Ethics Committee of Fuwai Hospital (License no.: FW-2022-0016).

3.1 Effects of dietary salt on isovalerylcarnitine in rats

To validate the effects of dietary salt on the key SSBP-related metabolites (i.e., isovalerylcarnitine), we performed an intervention trial in rats. Four-week-old male Wistar rats (Beijing HFK Bioscience Co., Ltd, China) were randomly allocated to the high-salt diet (HSD) group (n = 15) and the normal-salt control group (n = 15). Feeds for these two groups were purchased from Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd. They all were gamma-irradiated (25 kGy), and their components were identical except for sodium chloride (NaCl) content [HSD: 8% NaCl (cat#XTNaCl-8C), control: 0.45% NaCl (cat#1010088)]. The components of feeds are listed below:

Component	Control diet	HSD
Energy density, kcal/kg	3,790	
Protein, %	21.5	
Fat, %	11.1	
Carbohydrate, %	67.4	
NaCl, g/kg	4.5	80
Protein source	Fish meal, Chicken meal, Soybean meal	
Fat source	Soybean oil	
Carbohydrate source	Corn, Wheat	
Vitamin	Vitamin A, Vitamin D3, α-tocopheryl acetate, Menadione Sodium bisulfite, Thiamine nitrate, Riboflavin, Pyridoxine hydrochloride, Cyanocobalamin, Niacinamide, D-calcium pantothenate, Folic acid, D-biotin	
Mineral and other micronutrient	Limestone powder, Dicalcium phosphate, Choline chloride, Tribasic copper chloride, Ferrous sulfate, Manganese sulfate, Hydroxy methionine analog chelated zinc, Selenium yeast	

Before the intervention, all rats were fed using a control diet for one week, and a tail-cuff system (Softron BP-2010 Series; Softron, China) was used to perform adaptive training for them. After that, a four-week intervention was conducted. BP of each rat was measured at the end of per week in the daytime using the tail-cuff system. Each rat was measured for multiple readings until BP was stable, and the last three measurements were used to calculate the average level of BP for the corresponding time point. Blood samples of each rat were collected at the end of the fourth week, and stored in -80 °C refrigerators immediately.

3.2 Effects of isovalerylcarnitine on SS hypertension in rats

To confirm the protective effect of isovalerylcarnitine on SS hypertension, we performed an experiment in Dahl SS rats. Seven-week-old male rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (China). Before the intervention, rats were given a normal-salt diet containing 0.45% NaCl. Hypertension was induced by HSD containing 8% NaCl. Feeds were the same as those used in the Wistar rats (diet with 8% NaCl: cat#XTNaCl-

8C, and diet with 0.45% NaCl: cat#1010088). Dahl SS rats were randomly assigned to three groups: group A [control (0.45% NaCl), n = 7], group B [HSD (8% NaCl), n = 11], and group C [HSD (8% NaCl) + isovalerylcarnitine, n = 11]. Rats in group C received a subcutaneous infusion of isovalerylcarnitine (MedChemExpress, Cat#139144-12-0) dissolved in saline at a dose of 200 μg/kg/d, using an osmotic mini-pump (Alzet Model 2004; DURECT, Cupertino, CA, USA) for 28 days. Rats in groups A and B were implanted osmotic mini-pump containing saline. Rats were surgically implanted with radiotelemetry (HD-X10, Data Sciences International, New Brighton, Minnesota) to monitor BP levels as described previously[8]. The catheter was placed into the femoral artery of rats. The surgery in rats was conducted under anesthesia induced by 4% isoflurane and maintained at 2% isoflurane. Post-surgery, rats were housed individually and allowed to recover for at least three days before measuring BP. SBP and DBP were continuously recorded from 10 pm to 1 am on the monitoring day using the Ponemah v6.50 software, and the average BP was calculated for analysis. Isovalerylcarnitine supplementation and HSD intervention were initiated after the baseline BP measurement. BP was recorded on days 3, 7, 14, 21, and 28. At the end of the experiment, rats' blood, urine, and tissue specimens were collected.

The vasodilatation was measured as previously described[9]. The mesenteric arteries of rats were rapidly dissected free from surrounding tissues, dissected into 2 mm segments, and mounted on pins between the micrometer and force transducer. Experiments were performed in organ chambers (DMT 620M, Aarhus, Denmark) containing Krebs buffer (in mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 11.1 glucose, 2.5 $CaCl_2$, and 0.03 EDTA-Na_2. Each artery ring was bathed at 37 ℃ and continuously bubbled with 95% O_2 and 5% CO_2. The tone of each vessel was recorded using LabChart v8.1.13 (AD Instruments, Australia). The maximal reference tension was tested using Krebs buffer with high K^+, composed of (in mM): 63 NaCl, 60 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 11.1 glucose, 2.5 $CaCl_2$, and 0.03 EDTA-Na_2. The arteries were contracted using phenylephrine to produce 80% of the maximum contraction. Increasing concentrations of acetylcholine or sodium nitroprusside were used to assess endothelium-dependent or endothelium-independent relaxation of mesenteric arteries, and the response to each concentration of the drug was recorded.

For histological analysis, aortas and kidneys were harvested post-sacrifice and fixed in 4% (v/v) paraformaldehyde (PFA). Tissues were paraffin-embedded, sectioned at 4 μm thickness, and subjected to: Hematoxylin and eosin (H&E) staining for general morphology assessment; Masson's trichrome staining to quantify collagen deposition in tunica media of the thoracic

aorta; and Sirius red staining to detect collagen deposition in renal cortex[10]. Immunofluorescent staining was performed on thoracic aortas. Sections were blocked with 3% bovine serum albumin (1 h, RT), then incubated with mouse anti-eNOS antibody (Supplier: Santa Cruz; Cat: sc-376751; mouse mAb; RRID: AB_2832203) (1:300; 2 mg/mL) overnight at 4°C. After PBS washes (3 × 5 min), samples were incubated with Alexa Fluor 647-conjugated donkey anti-mouse IgG secondary antibody (1:500; 1 h, 37°C), washed again with PBS (3 × 5 min), and counterstained with DAPI for nuclear visualization[11].

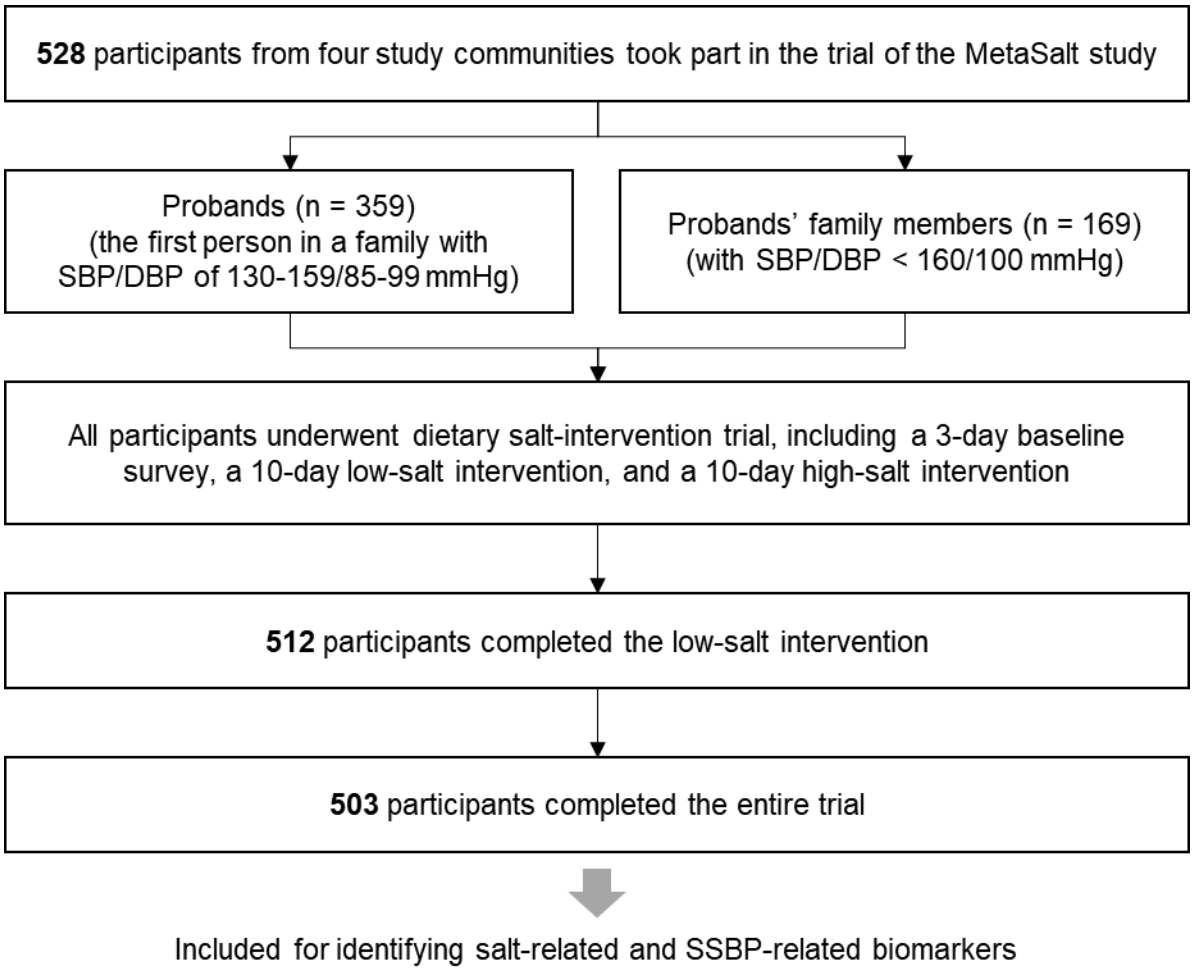
We also conducted an additional experiment by replacing subcutaneous administration of isovalerylcarnitine with oral administration. To determine an oral dosage, we referred to previous studies involving carnitine or acylcarnitines[12,13], and performed a pharmacokinetic analysis. Six rats received either 2 mg/kg orally or 200 μg/kg subcutaneously (n = 3 per group), and plasma concentration-time curves over 0-4 hours were comparable ($p = 0.2480$) (Supplementary Fig. 14a), indicating that 2 mg/kg orally approximates the plasma concentration achieved by subcutaneous injection. For the subsequent intervention, the compound was delivered via drinking water. Based on an average body weight of 0.3 kg and daily water intake of 80 ml, the required concentration was calculated as 7.5 μg/ml. After pharmacokinetic analysis, 20 seven-week-aged male Dahl SS rats were randomly allocated to group A: control group (0.45% NaCl, n = 4), group B: HSD group (8% NaCl, n = 8), and group C: HSD + isovalerylcarnitine (8% NaCl in diet and 7.5 μg/ml isovalerylcarnitine added in drinking water). All rats were fed using a control diet for a week. A tail-cuff system was used to perform adaptive training, and then a four-week intervention was conducted. BP levels were measured on days 0, 7, 14, 21, and 28 using the tail-cuff system. Each rat was measured for multiple readings until BP was stable, and the last three measurements were used to calculate the average level of BP for the corresponding time point.

3.3 Validation of microbiota source for isovalerylcarnitine

To verify that isovalerylcarnitine could be produced by gut microbiota, we conducted a 28-day experiment of gut microbiota suppression and fecal microbiota transplantation (FMT) in 21 six-week-old Sprague-Dawley rats (Beijing HFK Bioscience Co., Ltd, China). All rats were fed with diet containing 0.45% NaCl (cat#1010088). These rats were randomly allocated to three groups, including the control group (without any intervention, n = 7), the group with antibiotic cocktail treatment (ACT) (n = 7), and the group with ACT and subsequent FMT (n = 7) (Supplementary Fig. 15a). The antibiotic cocktail was composed of ampicillin (1 g/L), vancomycin (0.5 g/L), metronidazole (1 g/L), and neomycin (1 g/L), all purchased from

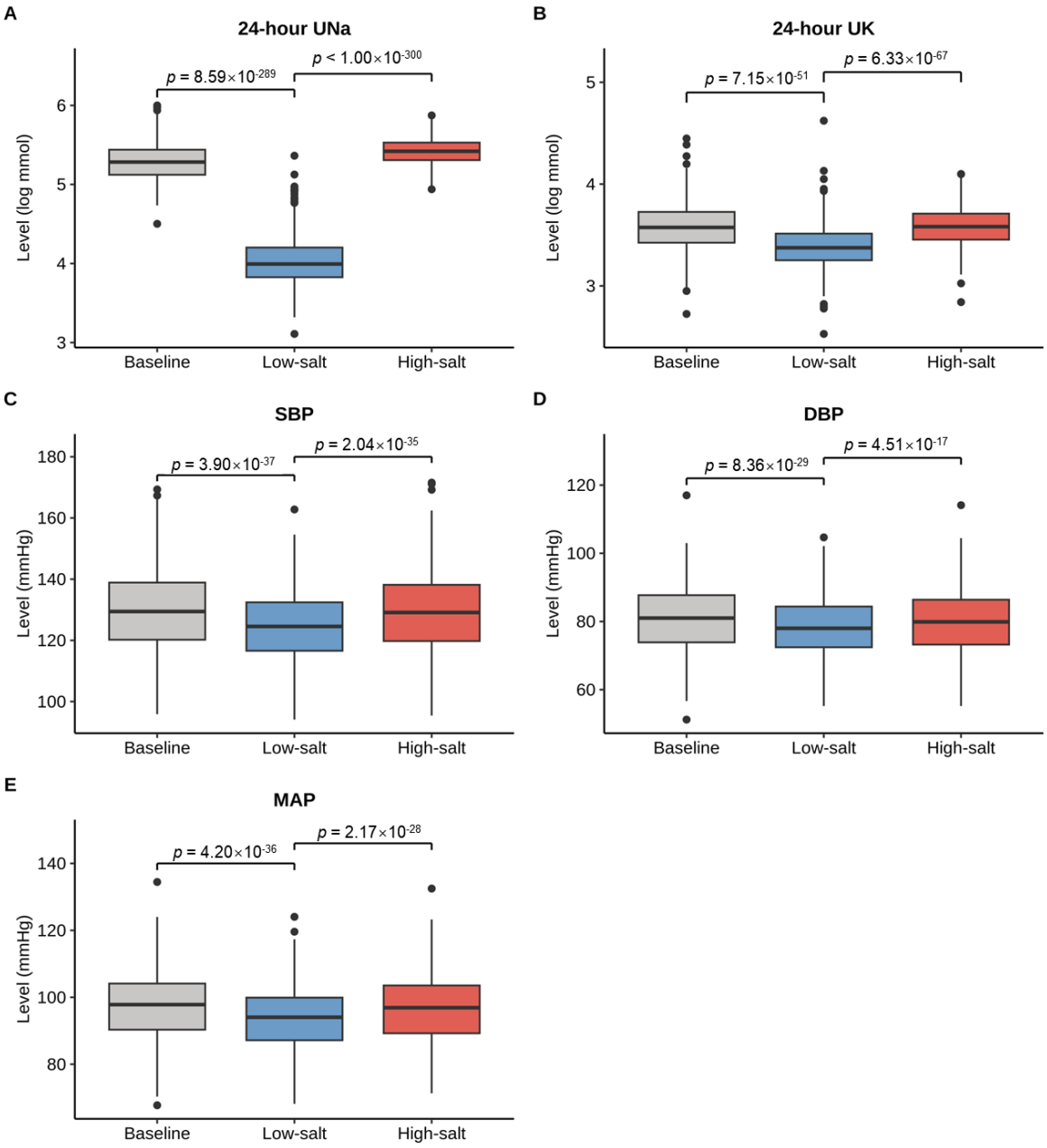
MedChemExpress. The antibiotics were administered in the drinking water of the rats in the ACT and ACT to FMT groups to decrease the bacterial load[14]. Water was replaced daily, and bottles were wrapped in foil to protect them from light. For FMT preparation, fresh fecal samples from control rats were collected using sterile tools, suspended in phosphate buffer saline (PBS) containing 0.05% L-cysteine hydrochloride at a ratio of 1:5 (w/v), and then filtered through a 70 μm strainer to obtain a bacterial suspension. All preparation steps were performed in an anaerobic chamber. Rats in the ACT to FMT group were orally gavaged with 1 ml of the filtrate once daily for one week following ACT. One rat in the ACT + FMT group was scarificed during the intragastric administration procedure. Blood and fecal samples were collected to detect metabolites and gut microbiota using UPLC-MS/MS and 16S amplicon sequencing, respectively. Amplicon sequence variant (ASV) richness was used to assess bacterial clearance and rebuilding.

Supplementary Figures



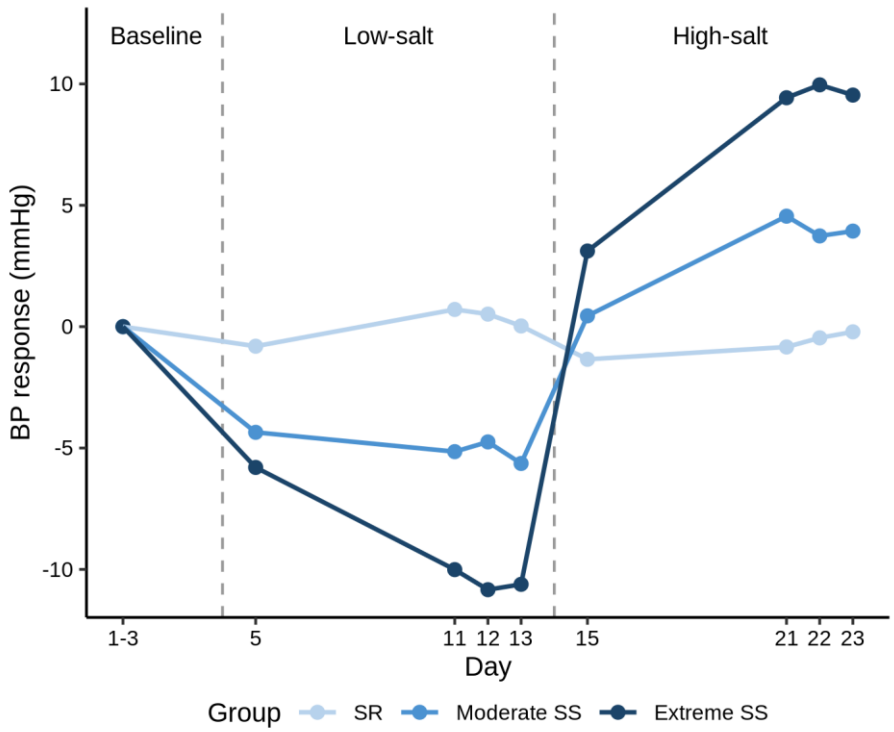
Supplementary Figure 1. Flow chart of participants' enrollment and participation in the MetaSalt study

DBP, diastolic blood pressure; SBP, systolic blood pressure.



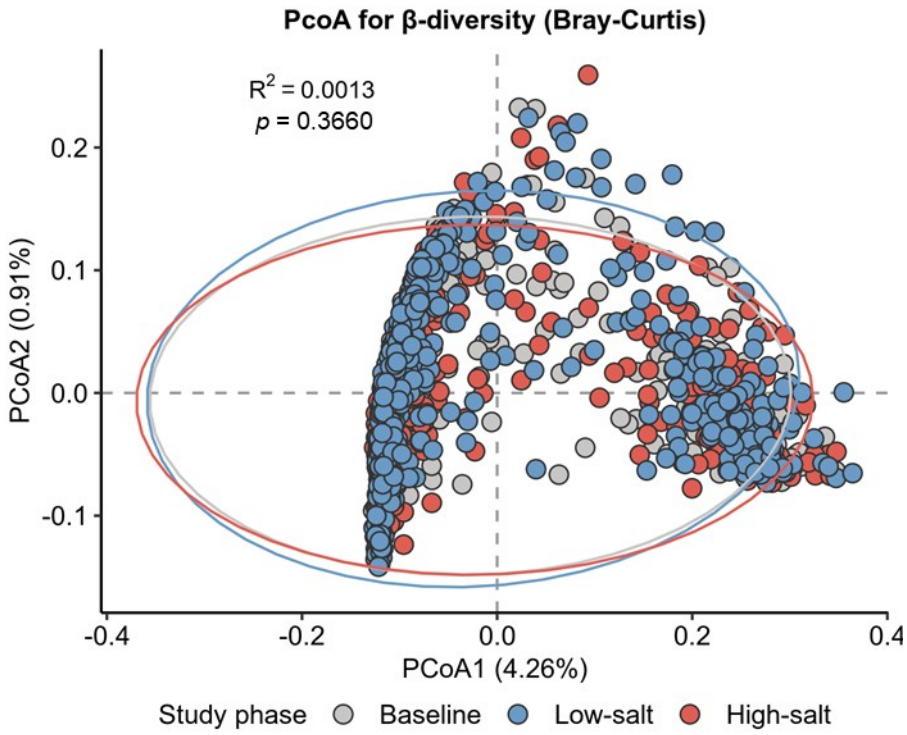
Supplementary Figure 2. Effects of dietary salt intervention on 24-hour UNa, 24-hour UK, and BP

Differences were tested using linear mixed models with the random intercepts of individuals and families among the MetaSalt participants (n = 503). In each boxplot, the center line indicates the median, the box represents the interquartile range (IQR), the whiskers extend to the minimum and maximum values within 1.5 IQR, and points beyond the whiskers are outliers. *p*-values are two-sided. BP, blood pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure; UK, urinary potassium; UNa, Urinary sodium.



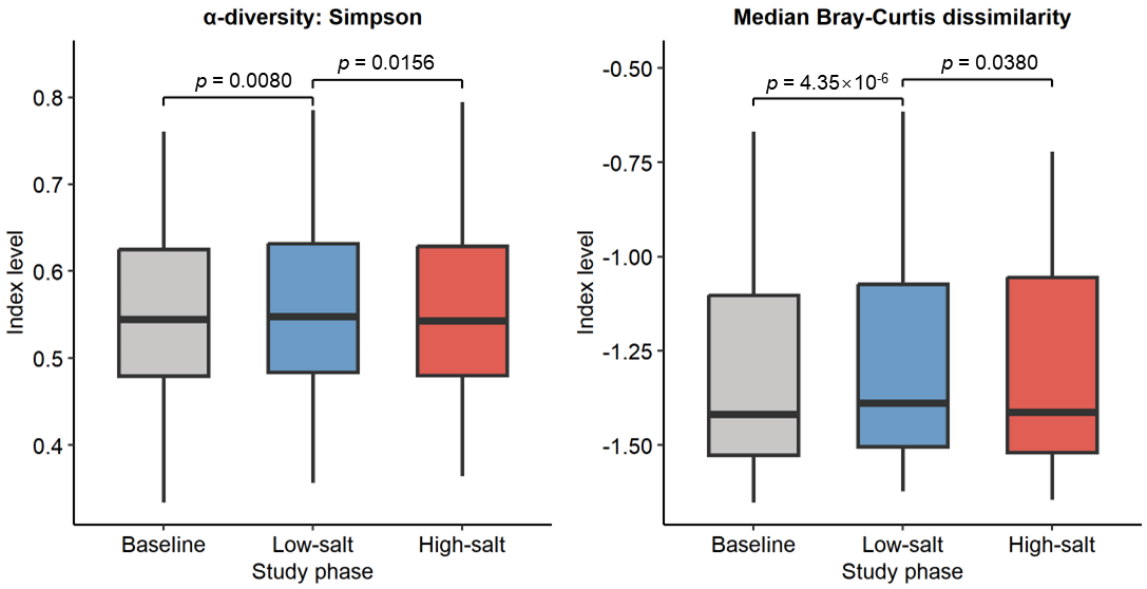
Supplementary Figure 3. MAP responses to the salt interventions across SSBP groups

Each single-day response of office MAP in the MetaSalt participants (n = 503), i.e., the changes in MAP from the baseline (mean MAP of the 3-day baseline) to days 2, 8, 9, 10 of the low-salt intervention, and the changes from the low-salt intervention (mean MAP of the last 3 days in the low-salt intervention) to days 2, 8, 9, 10 of the high-salt intervention were calculated. The average MAP responses at each time point were calculated separately for each group. MAP, mean arterial pressure; SR, salt resistance; SS, salt sensitivity; SSBP, salt sensitivity of blood pressure.



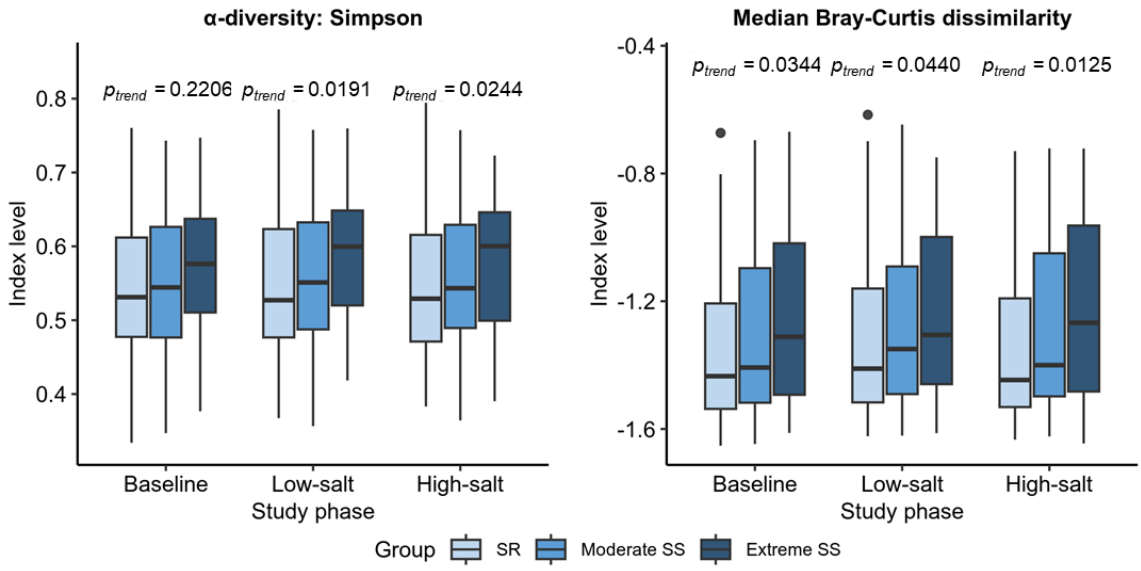
Supplementary Figure 4. PCoA plot of β-diversity for study phases

β-diversity was assessed for the MetaSalt participants (n = 503) based on species-level Bray-Curtis distance. The first two PCoAs could explain 4.26% and 0.91% of the variance in β-diversity, respectively. Difference in β-diversity was tested using permutational analysis of variance (999 permutations). *p*-values are two-sided. PCoA, principal coordinate analysis.



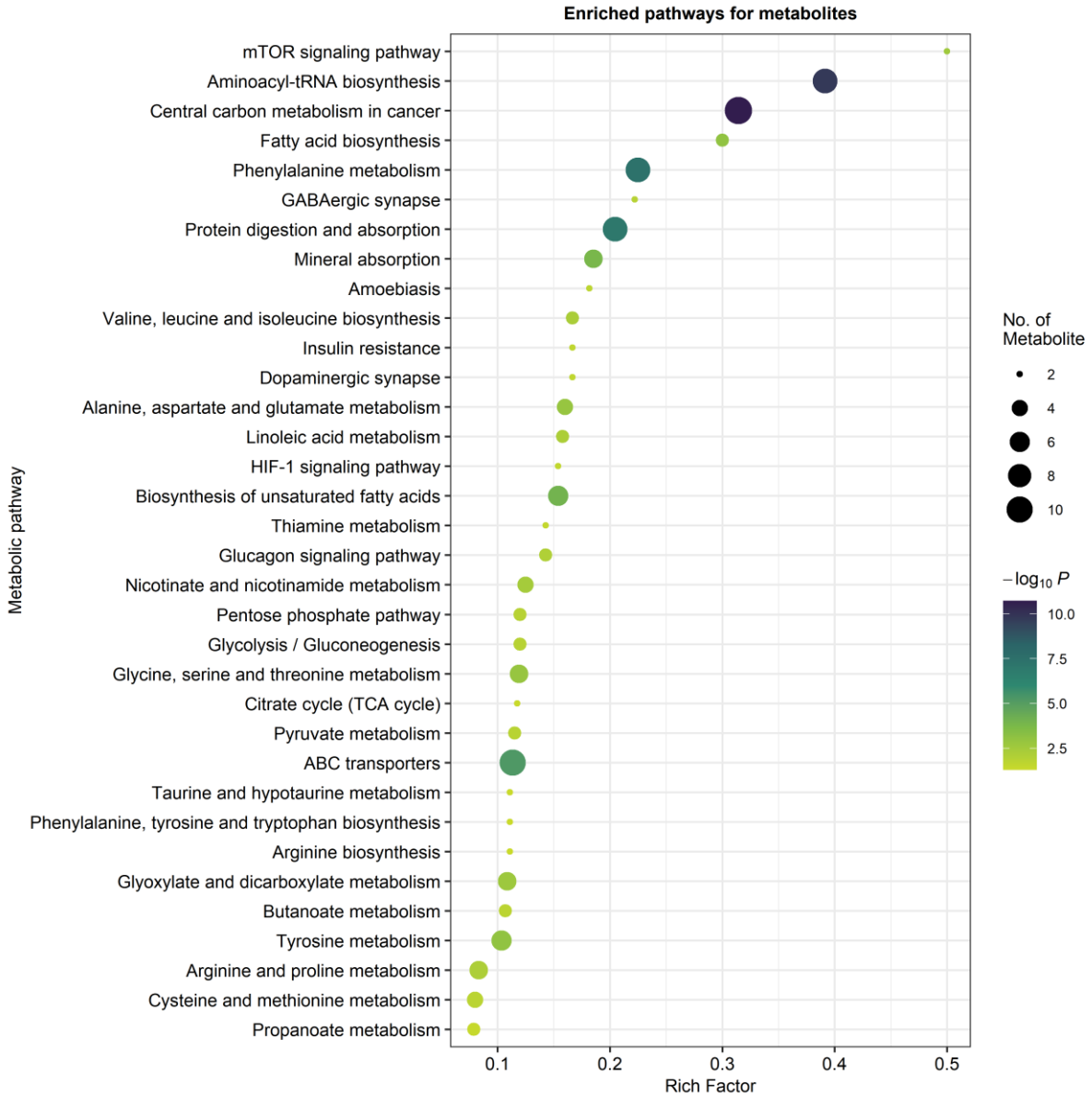
Supplementary Figure 5. α-diversity and median Bray-Curtis dissimilarity across study phases

Gut-microbial diversity was assessed for the MetaSalt participants (n = 503). α-diversity was estimated based on Simpon index. Median Bray-Curtis dissimilarity for each individual in each study phase was calculated as the median of distance measures against all other individuals, and log-transformed before analysis due to skewed distribution. Differences were tested using linear mixed models with the random intercepts of individuals and families. In each boxplot, the center line indicates the median, the box represents the interquartile range (IQR), the whiskers extend to the minimum and maximum values within 1.5 IQR, and points beyond the whiskers are outliers. *p*-values are two-sided.



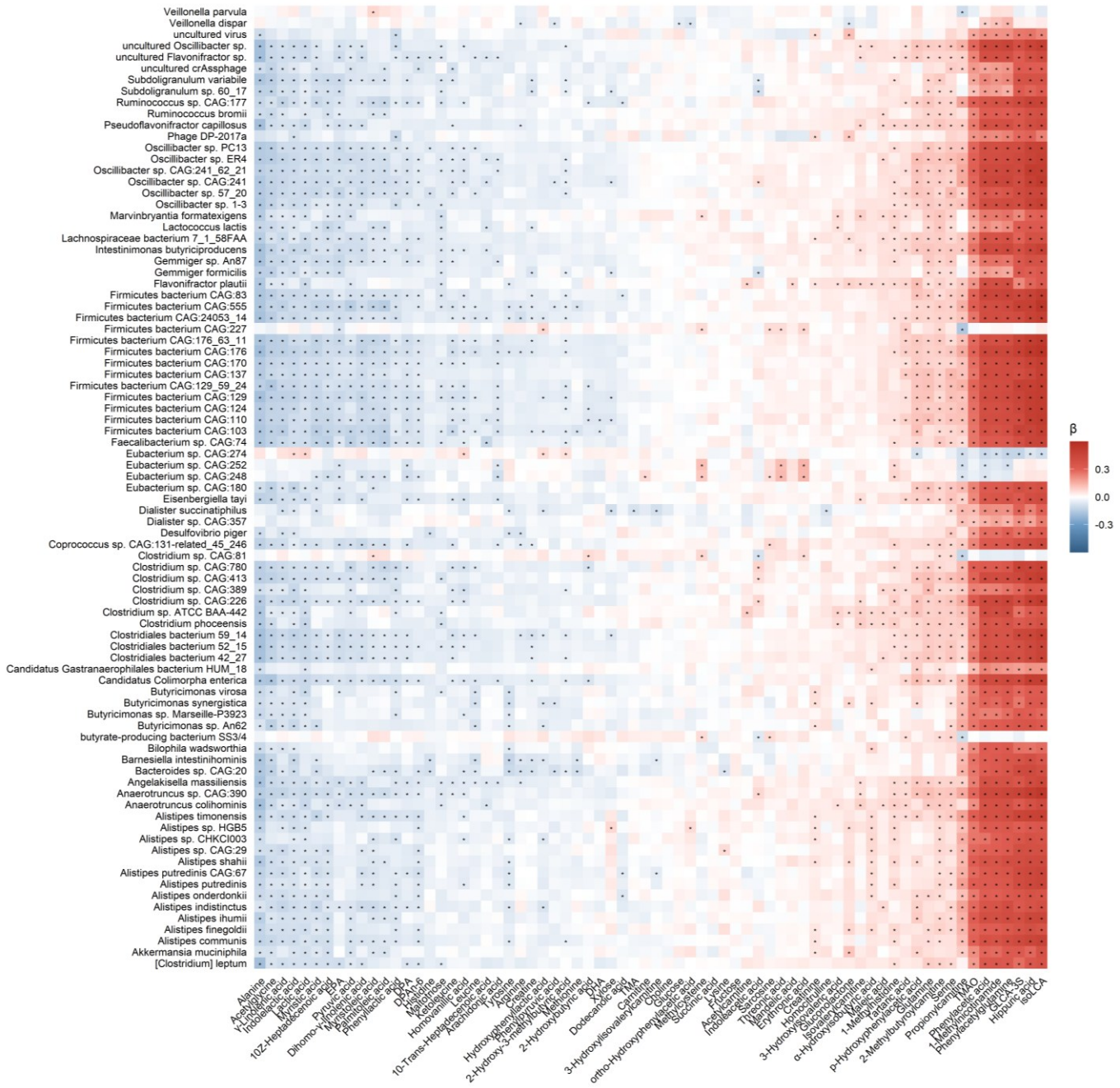
Supplementary Figure 6. α-diversity and median Bray-Curtis dissimilarity across SSBP groups in each study phase

Gut-microbial diversity was assessed for the MetaSalt participants (n = 503). α-diversity was estimated based on Simpon index. Median Bray-Curtis dissimilarity for each individual in each study phase was calculated as the median of distance measures against all other individuals, and log-transformed before analysis due to skewed distribution. Differences were tested using linear mixed models with the random intercepts of individuals and families. In each boxplot, the center line indicates the median, the box represents the interquartile range (IQR), the whiskers extend to the minimum and maximum values within 1.5 IQR, and points beyond the whiskers are outliers. *p*-values are two-sided. SS, salt sensitivity; SSBP, salt sensitivity of blood pressure; SR, salt resistance.



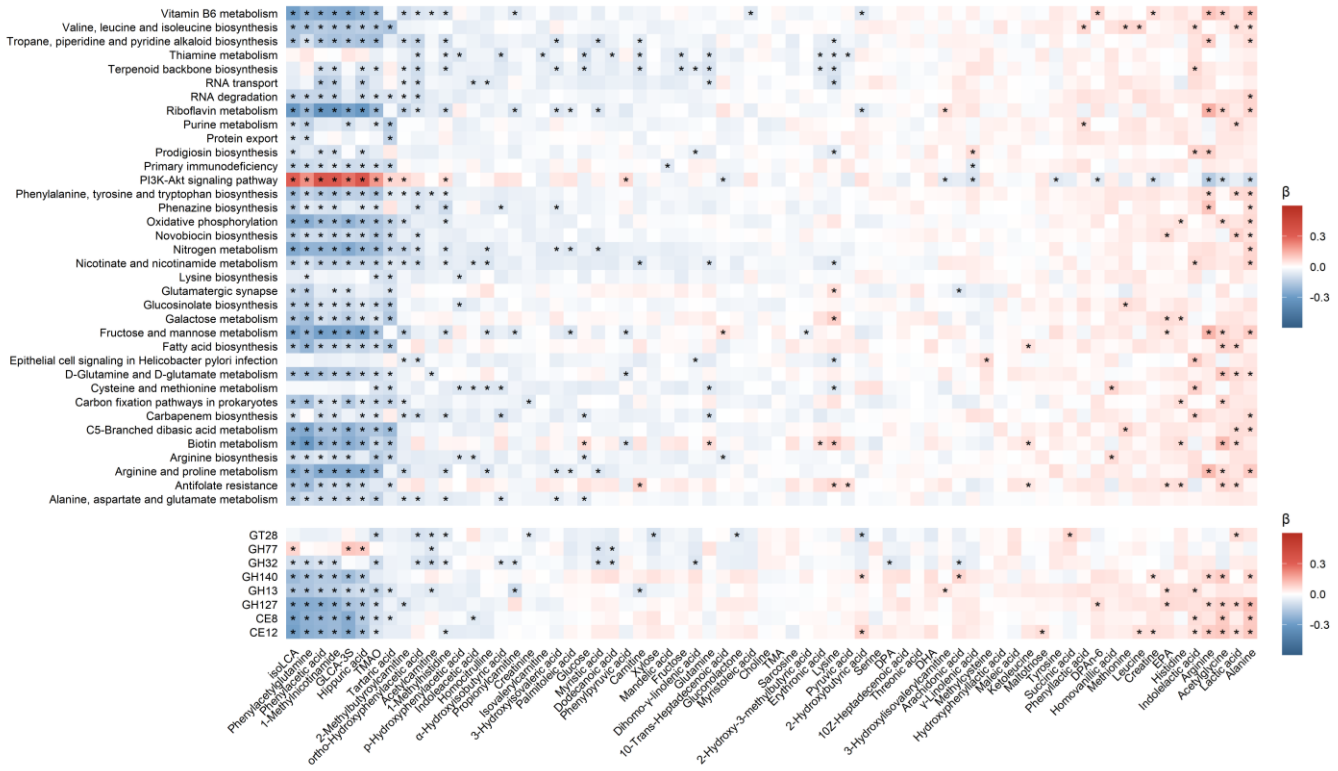
Supplementary Figure 7. Enriched KEGG pathways identified based on salt-related metabolites

Enriched KEGG pathways were identified based on salt-related metabolites using pathway over-representation analysis, and these pathways were considered to be significantly different between the low-salt and high-salt interventions ($p < 0.05$). *p*-values are two-sided. KEGG, the Kyoto Encyclopedia of Genes and Genomes database.



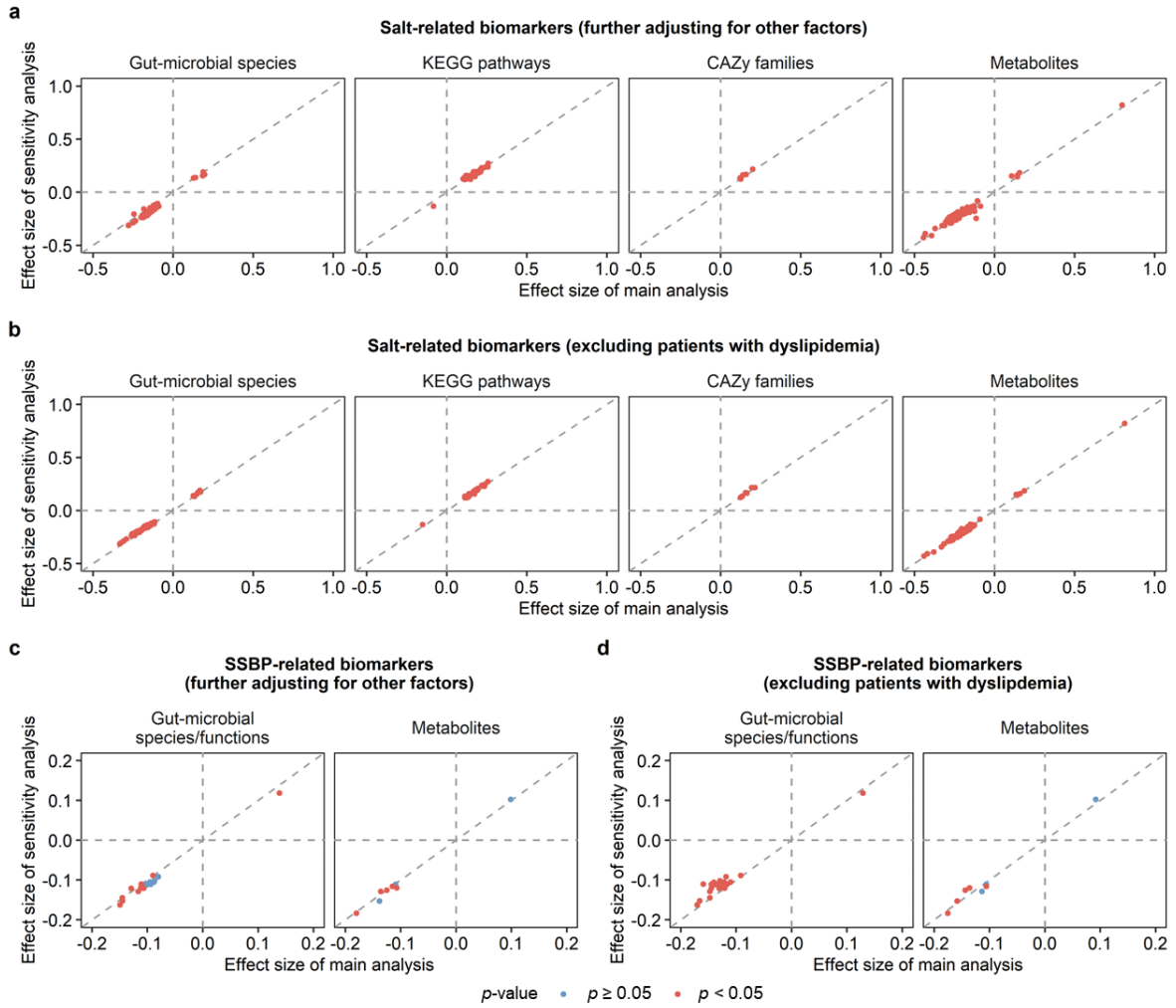
Supplementary Figure 8. Associations between salt-related gut-microbial species and salt-related metabolites

Associations were assessed using linear mixed models with the random intercepts of individuals and families among the MetaSalt participants (n = 503), adjusted for age, sex, field centers, body mass index, smoking, hypertension, total cholesterol, and study phases. Associations with $p < 0.05$ were kept and labeled as "*" in the figure. *p*-values are two-sided. DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLCA-3S, glycolithocholic acid 3-sulfate; isoLCA, isolithocholic acid; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.



Supplementary Figure 9. Associations between salt-related gut-microbial functions and salt-related metabolites

Associations were assessed using linear mixed models with the random intercepts of individuals and families among the MetaSalt participants (n = 503), adjusted for age, gender, field centers, body mass index, smoking, hypertension, total cholesterol, and study phases. Associations with $p < 0.05$ were kept and labeled as "*" in the figure. *p*-values are two-sided. CAZy, the Carbohydrate-Active EnZymes database; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLCA-3S, glycolithocholic acid 3-sulfate; isoLCA, isolithocholic acid; KEGG, the Kyoto Encyclopedia of Genes and Genomes database; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.



Supplementary Figure 10. Sensitivity analyses for the identification of salt-related and SSBP-related biomarkers

Sensitivity analyses were performed to test the robustness of our findings regarding salt-related and SSBP-related biomarkers (n = 503 participants).

For **salt-related biomarkers** [including gut-microbial species, microbial functions (KEGG pathways and CAZy families), and metabolites], we conducted two sensitivity analyses:

a. adjusting for cardiometabolic risk factors (BMI, MAP, TC, and glucose levels) during both the low-salt and high-salt interventions, and

b. excluding participants with dyslipidemia to assess the influence of pre-existing metabolic conditions.

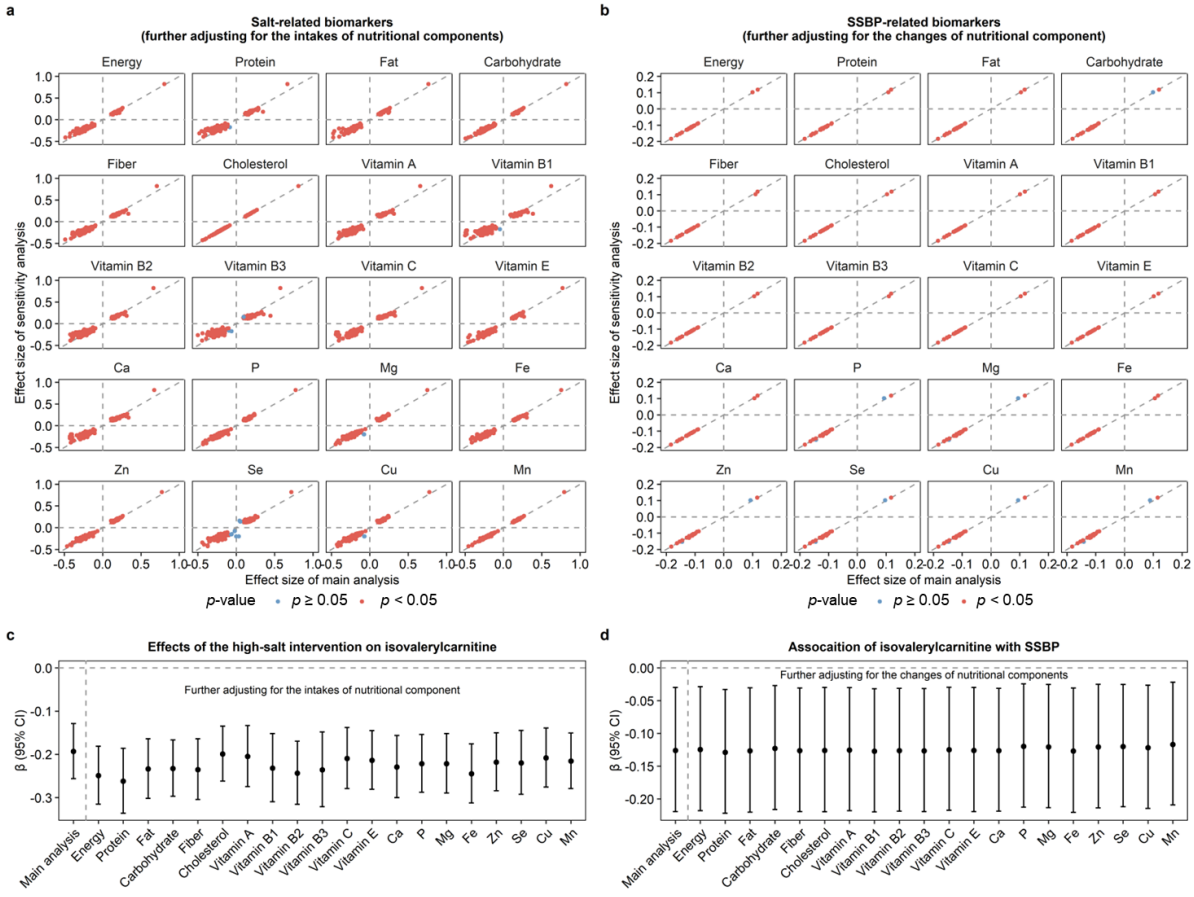
For **SSBP-related biomarkers**, we further conducted:

c. additional adjustment for potential baseline confounders, including dietary score, work-related physical activity, alcohol consumption, education, and eGFR, and

d. exclusion of individuals with dyslipidemia.

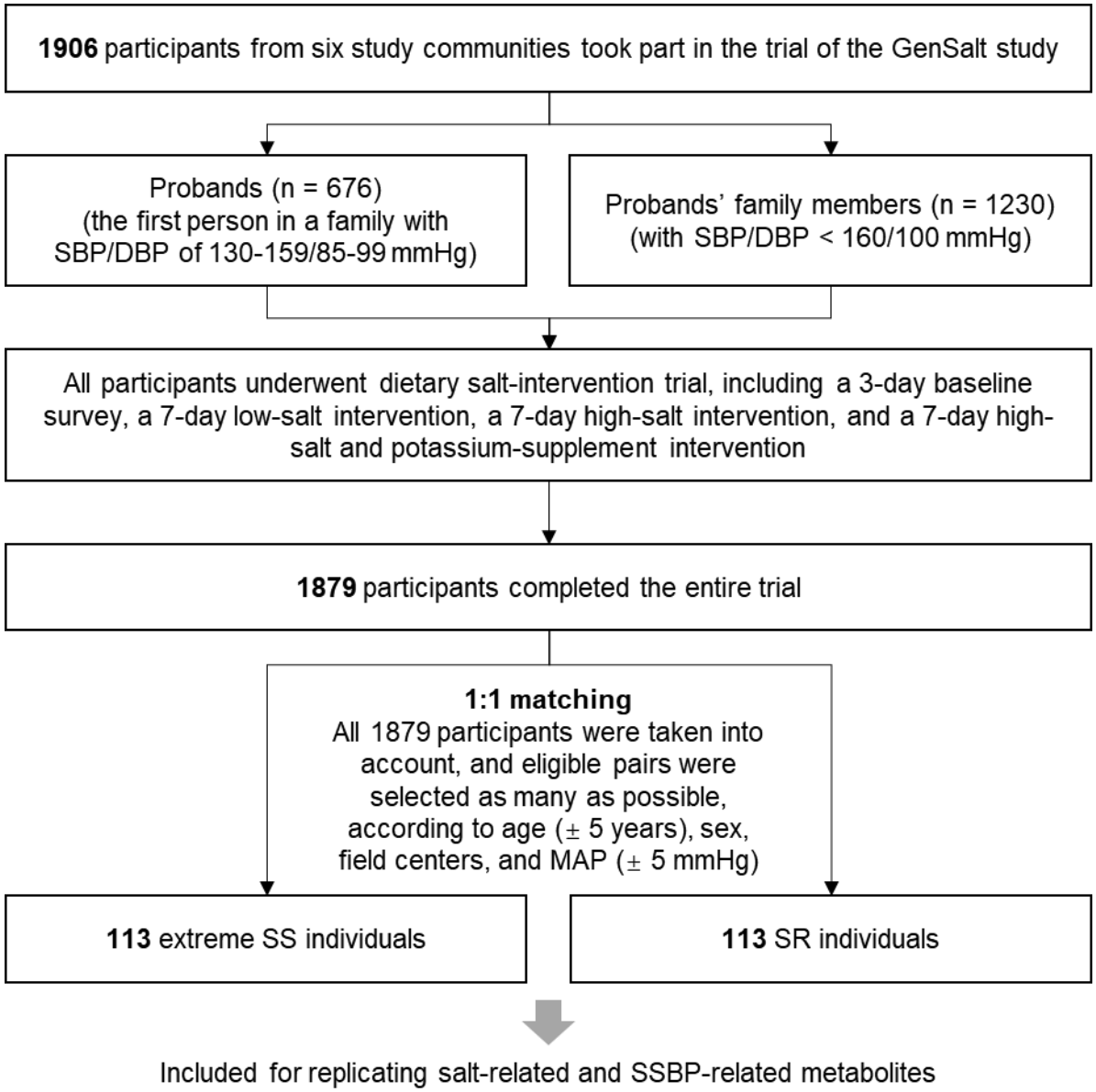
All analyses were conducted using linear mixed model. Effect sizes from the main and sensitivity analyses were visualized as scatter plots. Red dots indicate associations that remained significant ($p < 0.05$) in sensitivity analyses, while blue dots indicate those that did not ($p \geq 0.05$). *p*-values are two-sided.

BMI, body mass index; CAZy, the Carbohydrate-Active EnZymes database; KEGG, the Kyoto Encyclopedia of Genes and Genomes database; MAP, mean arterial pressure; TC, total cholesterol; SSBP, salt sensitivity of blood pressure.



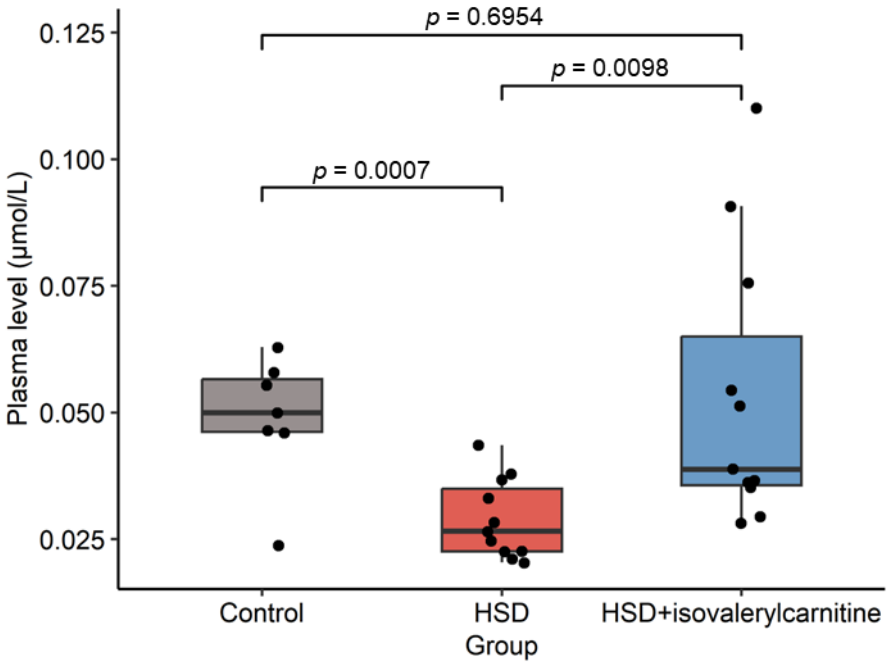
Supplementary Figure 11. Sensitivity analyses of further adjusting for nutritional components during the intervention stages

The intakes of energy and nutrients in the low-salt and high-salt intervention periods were calculated for the MetaSalt participants (n = 503). Sensitivity analyses of further adjusting for total energy or each nutrient were conducted using linear mixed model, which aimed to evaluate whether the effects of dietary salt on biomarkers and the associations of biomarkers with SSBP were confounded by other nutritional components. **a and b,** The effect sizes of sensitivity analyses and main analyses for all salt-related and SSBP-related biomarkers. The effect sizes estimated in the main analyses and the sensitivity analyses were illustrated as scatters, with red and blue dots indicating $p < 0.05$ and $p \geq 0.05$ in the results of the sensitivity analyses, respectively. **c and d,** The effect sizes of sensitivity analyses and main analyses for isovalerylcarnitine. *p*-values are two-sided. Data are presented as effect size (95% CI).



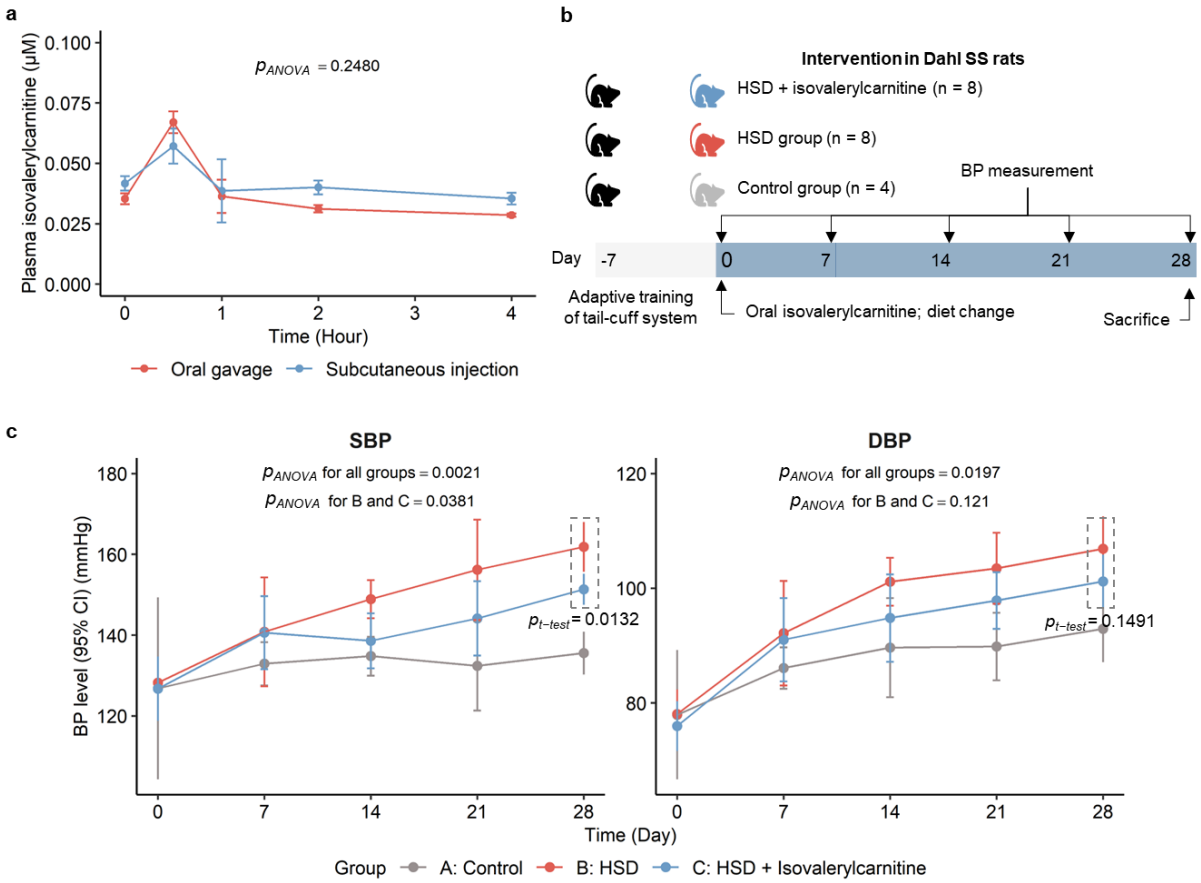
Supplementary Figure 12. Flow chart of participants' enrollment and participation in the GenSalt study

DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure; SS, salt sensitivity; SR, salt resistance.



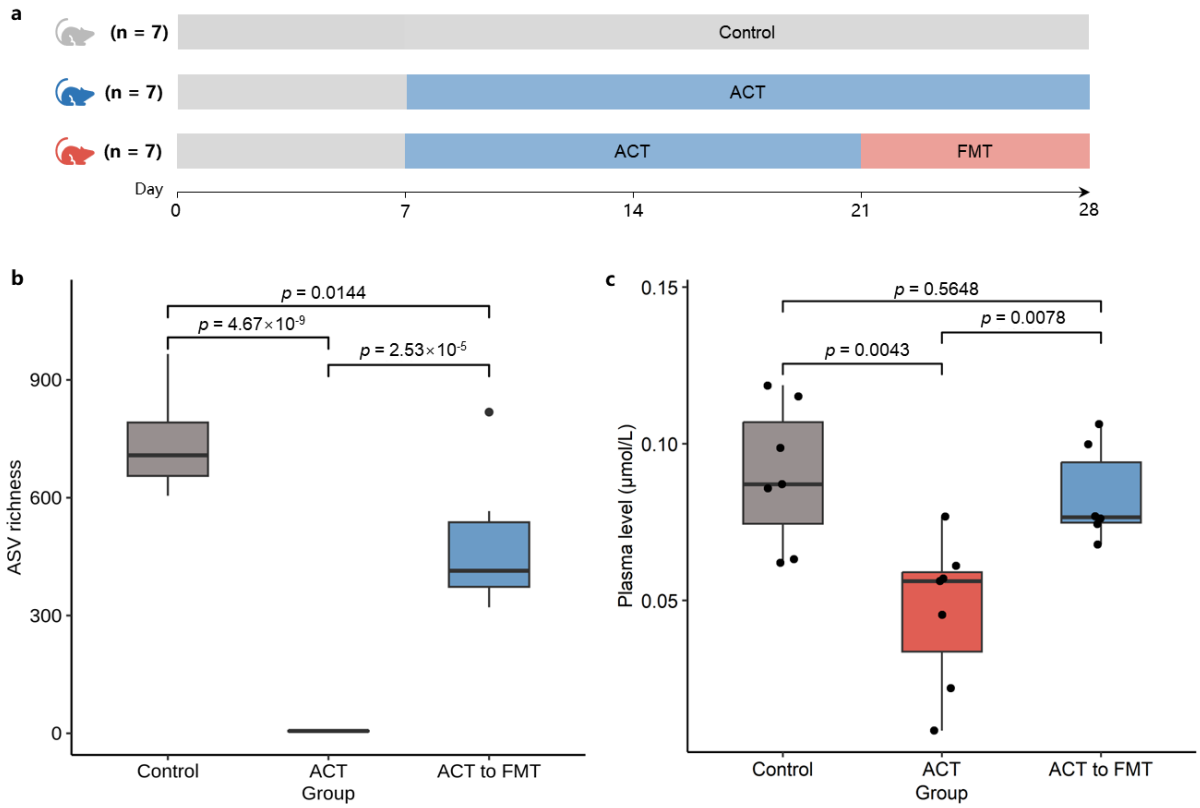
Supplementary Figure 13. Differences in isovalerylcarnitine among different groups of Dahl SS rats

Twenty-eight Dahl SS rats were randomly allocated to three groups: control (n = 7), HSD (n = 11), and HSD + isovalerylcarnitine (n = 11) groups. Differences between groups were tested using t-test. *p*-values are two-sided. In each boxplot, the center line indicates the median, the box represents the interquartile range (IQR), the whiskers extend to the minimum and maximum values within 1.5 IQR, and points beyond the whiskers are outliers. HSD, high-salt diet; SS, salt sensitivity.



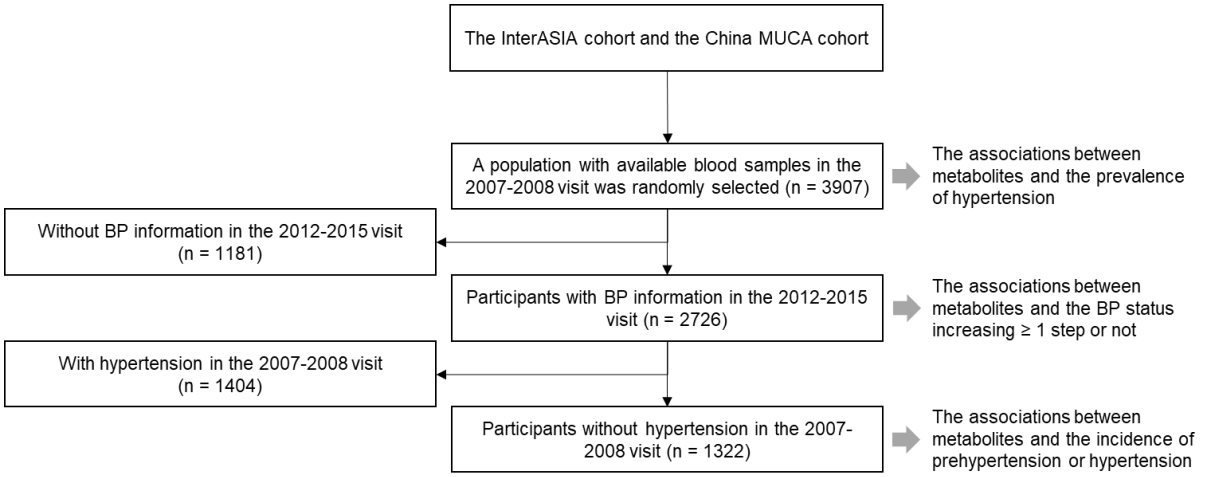
Supplementary Figure 14. Schematic diagram and results of the intervention experiment of oral isovalerylcarntine in Dahl SS rats

a, Plasma concentrations of isovalerylcarnitine after oral gavage (2 mg/kg) or subcutaneous injection (200 μg/kg) in a pharmacokinetic analysis (n = 6 animals). **b**, Schematic diagram of the intervention experiment of oral isovalerylcarnitine. **c**, Differences in BP levels between groups. Twenty Dahl SS rats were randomly allocated to three groups: control (n = 4), HSD (n = 8), and HSD + oral isovalerylcarnitine (n = 8) groups. Differences among groups were tested using repeated-measurement ANOVA or t-test. *p*-values are two-sided. Data are presented as effect size (95% CI). ANOVA, analysis of variance; DBP, diastolic blood pressure; HSD, high-salt diet; SBP, systolic blood pressure.



Supplementary Figure 15. Schematic diagram and the results of the intervention experiment of gut microbiota suppression and fecal microbiota transplantation in Sprague-Dawley rats

a, Schematic diagram of the intervention experiment of gut microbiota suppression and fecal microbiota transplantation. **b,** Differences in ASV richness among different groups. **c,** Differences in isovalerylcarnitine among different groups. Twenty-one Sprague-Dawley rats were randomly allocated to three groups: control (n = 7), ACT (n = 7), and ACT to FMT (n = 7), and one rat in the ACT + FMT group was sacrificed during the intragastric administration procedure. Differences between groups were tested using t-test. *p*-values are two-sided. In each boxplot, the center line indicates the median, the box represents the interquartile range (IQR), the whiskers extend to the minimum and maximum values within 1.5 IQR, and points beyond the whiskers are outliers. ACT, antibiotic cocktail treatment; ASV, amplicon sequence variant; FMT, fecal microbiota transplantation.



Supplementary Figure 16. Inclusion and exclusion criteria for the cohort used to assess the association of metabolite with hypertension

BP, blood pressure.

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