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Circulating metabolites, genetics and lifestyle factors in relation to future risk of type 2 diabetes

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Supplemental Materials

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Supplemental Methods

Study cohorts, data collection, and T2D ascertainment in each cohort

The Nurses' Health Study (NHS), NHSII, and Health Professionals Follow-up Study (HPFS): The NHS is an ongoing prospective cohort study in the US initiated in 1976 and enrolled 121,701 female registered nurses aged 30-55 years; the NHSII was initiated in 1989 and recruited 116,429 female nurses aged 25-42 years; and the HPFS was established in 1986 and similarly enrolled 51,529 men in health professions nation-wide aged 40-75 years.[1,2] Participants in all 3 cohorts completed a baseline questionnaire and have been followed up biennially. Diet was assessed every 4 years using a validated semi-quantitative food frequency questionnaires (FFQ).[3] T2D were identified by follow-up questionnaire, and confirmed through a supplementary questionnaire, based on the diagnosis criteria from the National Diabetes Data Group before 1998,[4] the criteria of the American Diabetes Association after 1998,[5] and further consideration of HbA1c $\geq$ 6.5% after January 2010.[6] A validation study showed that 97% of the T2D cases diagnosed through this protocol were confirmed by medical record review.[5,7]

Blood samples were collected from sub-populations of the NHS (n=32,826) during 1989-1990,[8] NHSII (n=29,611) during 1996-1999,[9] and HPFS (n=18,018) during 1993-1995 (with >95% follow-up rate).[10,11] Plasma metabolomics was measured using samples from the first blood collection by multiple sub-studies.[12] Our prospective analysis of plasma metabolites and incident T2D included 6,890 women from NHS, 3,692 women from NHSII, and 2,529 men from HPFS with qualified metabolomics data, and were free of diabetes, cardiovascular disease (CVD), and cancer at the time of blood draw. After up to 26 years of follow-up (through June 2016 in NHS, June 2017 in NHSII, and June 2016 in HPFS), 1,219 incident T2D cases in the NHS, 412 in NHSII, and 164 in HPFS, were identified.

Dietary intakes were assessed using a semi-quantitative food frequency questionnaire (FFQ) every 4 years. Baseline consumptions of 15 major food groups and an Alternate Healthy Eating Index -2010 (AHEI-2010; to assess overall dietary quality)[13] were calculated based on average dietary intakes from the two FFQs closest to the time of blood draw (NHS: 1986 and 1990; NHSII: 1995 and 1999; HPFS: 1994 and 1998). Medical conditions, family history, smoking history, and physical activity (metabolic equivalent MET-hours/week) were collected from biennial questionnaires preceding the blood collection. BMI was calculated based on weight from blood questionnaires; waist-hip ratio was based on data from biennial questionnaires closest to the blood collection. The eGFR was calculated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula, based on age, sex, and race, in subset of participants with serum creatinine data.[14] The Institutional Review Boards at Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health approved the study.

The Hispanic Community Health Study/Study of Latinos (SOL) is a prospective population-based study of 16,415 Hispanic/Latino adults aged 18-74 years living in 4 US metropolitan areas (Bronx, NY; Chicago, IL; Miami, FL; and San Diego, CA).[15,16] Comprehensive interviews and clinical assessments were conducted at in-person clinic visits during 2008-2011 (Visit 1) and 2014-2017 (Visit 2), collecting information on self-reported sex and other demographics, behavioral factors, health status, and medical histories, as well as blood pressure, anthropometric traits, and standard clinical biomarkers.[17] Annual follow-up telephone interviews was conducted

to collect health information. Incident T2D was defined when a participant was free of diabetes at baseline but was identified as having T2D during annual follow-up, which required meeting one of the following criteria including 1) fasting glucose ≥7.0 mmol/L; 2) fasting ≤8 hours and non-fasting glucose≥ 11.1 mmol/L; 3) post OGTT glucose ≥ 11.1 mmol/L; 4) HbA1c ≥6.5%; 5) current use of antidiabetic medications; or 6) self-reported physician diagnosed diabetes. Habitual dietary intake was estimated using the National Cancer Institute methodology based on two 24-h dietary recalls and a food propensity questionnaire, as described previously.[18] The eGFR was estimated based on standard reference equations for Hispanics and has been adjusted for age and sex. Our analysis of incident T2D included 2,821 individuals with metabolomics data and were free of CVD, cancer and diabetes at Visit 1. The study was approved by the institutional review boards at all participating institutions, and all participants gave written informed consent.

The Women's Health Initiative (WHI), launched in 1993, is a nationwide study for chronic disease prevention in postmenopausal women.[19] The study enrolled women aged 50-79 years old, including 68,132 women in one of three WHI clinical trials, and 93,676 women in the WHI Observational Study.[20] At the end of the initial study period in 2005, women who consented were continued to be followed up through WHI Extension Studies. Data on health and lifestyle related information were collected at baseline by trained staff, and during follow up through telephone interviews and annual clinical visit. Blood and urine samples were collected at baseline and some follow-up visits. T2D case was determined if a woman reported having a history of diabetes or using anti-diabetic medications (pills or shots) in visits/interviews. Incident T2D cases were defined as free of diabetes at baseline but became a T2D case during approximately 17 years of prospective follow up. Diet was assessed by an extensively-validated 122-item FFQ designed for application in multiethnic and geographically diverse populations.[21-23] The eGFR was estimated using the CKD-EPI equation based on age, sex, and race.[24] Our study utilized metabolomics data from a nested case-control study of coronary heart disease (CHD);[25] 1,392 women free of CVD, cancer, and diabetes, and were not in the active arms of the hormonal therapy trials, were included in analyses; 163 incident T2D cases were identified during follow-up. The study was approved by the institutional review board of Brigham and Women's Hospital, and all participants provided written informed consent.

The Atherosclerosis Risk in Communities (ARIC) Study is a community-based longitudinal study that recruited 15,792 mostly black and white US participants aged 45-64 years from 4 study centers (suburban Minneapolis, MN; Washington County, MD; Jackson, MS; and Forsyth County, NC). The initial enrollment was from 1987-1989, and six subsequent follow-up studies were completed from 1990 to 2019. At baseline and follow-up visits, trained staff collected information on self-reported sex and other demographics, socioeconomic status, health behaviors, and medical histories, and measured blood pressure and anthropometric traits. Blood samples were collected for clinical biomarker measurement[26,27] and future assays. Annual (semi-annual since 2012) follow-up telephone interviews were conducted to collect health information. Our prospective analyses of T2D risk included 1,288 White and 1,433 Black individuals with metabolomics data and were free of CVD, cancer, and diabetes at study baseline. T2D was diagnosed as meeting one of the following criteria: 1) fasting (>8 hours) glucose ≥7.0 mmol/L; 2) fasting ≤8 hours and non-fasting glucose≥ 11.1 mmol/L; 3) current use of antidiabetic

medications; or 4) self-reported physician diagnosed diabetes.[28] Incident T2D was defined as newly onset T2D from baseline through December 31 2015. The eGFR was calculated using the CKD-EPI formula considering age, sex, and race.[14,29,30] All study participants signed informed consent, the protocol was approved by the institutional review boards at all participating centers, and procedures were followed according to the Declaration of Helsinki.

The Framingham Heart Study Offspring cohort (FHS) is a community-based prospective cohort comprises 5,124 participants, and our study was based on 3,799 adults who attended the 5th quadrennial examination cycle during 1991-1995 (considered as the baseline in this study). Participants self-reported their demographic characteristics such as sex and age, clinical and lifestyle factors, and medical histories; underwent physician-administered physical examinations to collect weight, height, blood pressures, among others; and provided biological samples for routine laboratory tests.[31,32] Blood biomarkers were measured using standard methods. About 3,306 participants also provided dietary data assessed using a validated semiquantitative FFQ.[3] Specifically, our prospective analysis of T2D risk included 1,424 individuals with metabolomics data and free of CVD, cancer, and diabetes at study baseline. Incident T2D was ascertained at every quadrennial examination,[31] and participants who met at least one of the following criteria during follow-up were defined as T2D cases, including 1) fasting glucose ≥7.0 mmol/L; 2) fasting ≤8 hours and non-fasting glucose ≥11.1 mmol/L; or 3) current use of antidiabetic medications. All participants provided written informed consent, and the study protocol was approved by the Boston University Medical Campus Institutional Review Board.

The Multi-Ethnic Study of Atherosclerosis (MESA), initiated in 2000, enrolled 6,814 adults aged 45-84 years old who self-identified as white (38%), African American (28%), Hispanic (22%), or Chinese (12%), and were free of clinically diagnosed CVD from 6 study sites (New York, NY; Baltimore, MD; Forsyth County, NC; Chicago, IL; St Paul, MI; and Los Angeles, CA).[33-35] Participants' demographic (including self-reported sex, age, race and ethnicity) and psychosocial characteristics, family histories, medical histories, and anthropometric measures was collected at baseline (2000-2002), and time-varying information such as physical activity,[33] alcohol consumption, and smoking, was collected at baseline and updated during follow-up through questionnaires.[34,35] Four follow-up visits were conducted at an average of 1.6, 3.1, 4.8, and 9.5 years after baseline with high retention rate.[35] T2D was determined at baseline and at each examination according to the American Diabetes Association 2003 criteria,[36] which included fasting plasma glucose level ≥7.0 mmol/l or the use of oral antihyperglycemics or insulin.[34,35] A total of 902 participants free of T2D at baseline and had metabolomic data were included in our analysis, with new T2D cases identified at exams 2 to 4 considered as incident T2D. Written informed consent was obtained from the participants, and the study was approved by institutional review boards at each site.

The Boston Puerto Rican Health Study (BPRHS) is a prospective study that enrolled 1,500 self-identified Puerto Rican adults, aged 45–75 y, living in the greater Boston area, at baseline.[37,38] At baseline, and ~2 and ~6 year visits, bilingual, in-person interviews were conducted to collect information on sex, age, other demographic and psychosocial characteristics, medical and family histories, and lifestyle; trained staff measured

anthropometrics and blood pressures. Plasma clinical biomarkers were measured using standard assays.[37] Habitual diet was assessed with a semiquantitative FFQ designed and validated for the Puerto Rican population.[39] Physical activity was assessed using a modified Paffenbarger questionnaire from the Harvard Alumni Activity Survey.[40,41] Diabetes was defined as fasting glucose ≥7.0 mmol/l or reporting the use of antidiabetic medications. Participants who, at baseline, did not self-report a physician diagnosed diabetes, nor used antidiabetic medication, and had a fasting glucose <126 mg/dL, but were identified as having diabetes during ~6 years of follow-up visits, were considered as incident T2D cases. We included in our analysis, 378 participants who had baseline plasma metabolomics data, were free of diabetes and CVD at baseline, and attended 2-y or 5-y visits.[38] The study was approved by the institutional review boards at Tufts University, Northeastern University and the University of Massachusetts Lowell. All participants provided written informed consent.

The Prevención con Dieta Mediterránea Study (PREDIMED) is a 5-year multi-center randomized trial examining the efficacy of two Mediterranean diets versus a control diet (advice on a low-fat diet), for the primary prevention of CVD.[42] The study was initiated in 2003 and enrolled 7,447 participants aged 55-80 years who were at high risk of CVD. Participants' sex and other demographic characteristics were self-reported collected at baseline. At baseline and then annually, participants' medical histories and risk factors were collected through standard questionnaires, and anthropometric traits were measured by trained personnel. Adherence to the Mediterranean diet was assessed annually using a 14-item Mediterranean Diet Adherence Screener (MEDAS).[42] Baseline plasma biomarkers were assayed using standard methods, and eGFR was calculated using the CKD-EPI equation.[43] Two propensity scores were estimated based on 30 baseline variables to account for the probability of assignment to each of the intervention groups.[42] Incident T2D (a pre-defined secondary endpoint) was adjudicated through blind assessment by the Clinical Endpoint and Adjudication of Events Committee of PREDIMED, based on the criteria from the American Diabetes Association.[44] Among participants who were non-diabetic at baseline and had qualified blood samples, a nested case-cohort study was designed for metabolomic profiling, which comprised a randomly selected sub-cohort with 694 participants and 251 incident T2D cases identified through a median of 3.8 years follow-up (53 overleaping between sub-cohort and cases; details provided elsewhere).[45,46] After further excluding individuals with disqualified metabolomics data, 885 participants (including 248 incident T2D cases) were included in our analysis. Institutional Review Boards at each recruitment center approved the study protocol, and all the participants provided written informed consent.

Cardiovascular Health Study (CHS) is a NHLBI-sponsored prospective cohort study, originally enrolling 5,201 participants during 1989-1990, focusing on risk factors of CVD among older adults; a second cohort initiated in 1992-1993 and enrolled 678 predominantly black participants.[47-49] Our study only used GWAS summary statistics for plasma metabolites for 263 CHS participants of European ancestry whose metabolomics was measured using EDTA plasma samples from year 7. The study was approved by institutional review committees at each field center and individuals in the present analysis had available DNA and gave informed consent including consent to use of genetic information for the study of CVD.

Metabolomic profiling

Metabolomic profiling in a majority of our study cohorts was conducted with the Metabolomics Platform at the Broad Institute of M.I.T. and Harvard University (Cambridge, MA), using 3-4 complimentary liquid chromatography-tandem mass spectroscopy (LC-MS) methods. Detailed protocols have been described elsewhere.[25] The methods included a hydrophilic interaction liquid chromatography analysis of water-soluble metabolites in the positive ionization mode (HILIC-pos), which measures metabolites such as acylcarnitines, amino acids, peptides, and derivatives; a hydrophilic interaction liquid chromatography analysis of water-soluble metabolites (such as sugars, sugar phosphates, purine, and pyrimidines) in the negative ionization mode (HILIC-neg); a positive ion mode analysis (C8-pos) that measures polar and nonpolar plasma lipids; and/or a negative ion mode analyses of free fatty acids and bile acids (C18-neg). Throughout the assay, pooled plasma quality control samples were inserted every 20 samples, and results were standardized using the ratio of the value of the sample to the value of the nearest pooled reference multiplied by the median of all reference values for the metabolite. Quality control procedures were performed to ensure profiling quality and system performance.[25] As previously described, these four methods have been applied across various sub-studies in NHS/HPFS,[12] WHI,[25] and CHS; three methods (HILIC-pos, HILIC-neg, and C8-pos) have been applied to MESA,[50] PREDIMED,[25,45] and FHS.[31,51,52] Of all originally assessed metabolites from each cohort, 407 named metabolites from NHS, 363 from NHSII, 291 from HPFS, 364 from WHI, 327 from MESA, 274 from PREDIMED, and 188 from FHS, were harmonized and included in our T2D analysis (**Supplementary Table S1**). In CHS, 411 named metabolites were included in the genetic analysis (**Supplementary Table S7**).

Metabolomic profiling in SOL and ARIC (serum samples) and BPRHS (plasma samples) was conducted using the Metabolon DiscoveryHD4 Panel at the Metabolon Inc. (Durham, NC, USA), as described previously.[38,53,54] The Panel uses an untargeted approach with a Waters ACQUITY ultra performance LC system and a ThermoFisher Scientific Q-Exactive high resolution MS with a heated electrospray ionization source and Orbitrap mass analyzer.[55] Lab technicians were blinded to the sample IDs and characteristics; multiple internal quality control samples were inserted in a random order in all analyses. Standard Metabolon software was used to identify metabolites and perform standard data quality control and processing. After quality filtering in each cohort, 782 metabolites from SOL, 245 in ARIC, and 510 in BPRHS were retained, and eventually 283 metabolites in SOL, 139 in ARIC, and 231 in BPRHS were harmonized with other cohorts and analyzed in the study (**Supplementary Table S1**).

Metabolite harmonization across cohort and inclusion criteria for analysis

Within each study, basic quality filtering was conducted before analysis. Samples were removed if 1) their detection rate of metabolites is <80%, or 2) were outliers based on multidimensional scaling (MDS) analysis within a specific race/ethnic group. Metabolites were excluded if 1) their detection rate across all samples were <80%, and, if applicable, 2) coefficient of variation (CV) of the quality control samples was >20%. After quality filtering, for each metabolite, missingness were imputed using ½ minimum value; if the metabolomic profiling was performed through multiple batches (or sub-studies) within a cohort, the imputation was conducted separately within each batch (or sub-study). A total of 1,273 metabolites were initially qualified for analysis in at

least one cohort, measured by either the metabolomics platforms at the Broad Institute or Metabolon Inc. Across all cohorts, we then matched metabolites by their HMDB ID and/or PubChem ID, based on information provided by the corresponding metabolomic laboratories. To reduce single-study bias, we limited our final analyses to 469 metabolites that were available in ≥4 independent cohorts, or available in ≥3 independent cohorts if the 3 cohorts covered both the platforms at the Broad Institute and the Metabolon Inc.

Metabolome-wide association analysis for incident T2D

All association analyses were conducted in each cohort by major racial and ethnical groups (non-Hispanic White, Hispanics/Latinos, or African American) if the sample size in each group was sufficient for analysis (e.g., >95% NHS/HPFS participants were non-Hispanic White thus all NHS/HPFS participants were analyzed as one group; in MESA, analyses were done in two sets, non-Hispanic White individuals, and all those who were not non-Hispanic White, to ensure sufficient power). Detailed information on analysis sub-groups within each cohort and analytical models is presented in **Supplementary Table S2**.

Before analyses, metabolites were inverse normal transformed within each sub-study and racial/ethnic group (if applicable) in each cohort and to ensure a standard normal distribution (i.e., mean=0 and SD=1). For studies with a longitudinal cohort design (NHS excluding the T2D nested case-control sub-study; NHSII, HPFS, SOL, ARIC, WHI, FHS, MESA, and BPRHS), we used Cox regression to estimate hazards ratios (HRs) and 95% confidence intervals (CIs) of incident T2D per SD increment in metabolites; for NHS T2D nested case-control sub-study, we used logistic regression to estimate odds ratios (ORs) and 95% CI; and for the PREDIMED T2D nested case-cohort study, we applied a Cox regression with Barlow weights[56] and robust estimators to estimate HR and 95% CI. Five multivariable (MV) adjusted models were tested. A basic MV model (model 1) was adjusted for age in years, self-reported sex, smoking status, alcohol consumption, and if applicable, education, family income categories, fasting status, lipid-lowering medication use, anti-hypertensive medications, family history of diabetes, self-reported physician-diagnosed hypertension, self-reported physician-diagnosed dyslipidemia, as well as study specific basic covariates (**Supplementary Table S2)**. A model further adjusted for BMI and WHR (model 2) was used as the main model. In sensitivity analyses, model 1 was further adjusted for physical activity levels and dietary quality index (model 3); levels of HDL-cholesterol, LDL-cholesterol, and triglycerides (model 4); or systolic and diastolic blood pressures (model 5). In another sensitivity analysis, we further adjusted for eGFR on the basis of model 2 in NHS, NHSII, HPFS, SOL, ARIC, WHI, and PREDIMED. For each metabolite, we combined association results from all available cohorts and racial/ethnic groups using a fixed-effect, inverse variance weighted meta-analysis, and a meta-analyzed $FDR<0.05$ was considered statistically significant. In secondary analyses comparing results across racial/ethnic groups, meta-analysis was conducted combining results from the same racial/ethnic groups; when comparing results between the two platforms, meta-analysis was conducted combining cohorts using the same platforms.

To annotate the novelty of the identified associations, we conducted a literature review focusing on prospective cohort studies examining associations between circulating metabolites in relation to T2D risk. We used a literature review-based meta-analysis[57] that included all studies published before Mar 6, 2021 as an anchor point; and extracted information from this study and

then searched for additional studies published from 2021 to 2024.[58-72] Reported metabolites were matched to our study primarily by HMDB ID and then by name. We considered an association as "previously reported", if the association was statistically significant in a published study after multiple testing correction based on the study's pre-specified analysis plan.

GWAS of metabolites in each cohort

GWAS of metabolites have been previously conducted in the NHS/HPFS,[73] SOL,[74] ARIC,[74] WHI,[75] FHS,[76] and CHS, and the detailed analysis protocols in each cohort have been reported in previous publications.[73,74,76] We have summarized the key analytical details, including sample size, genotyping array and imputation methods, analysis models and tools, in **Supplementary Table S7**.

Briefly, in NHS/HPFS, genome-wide genotyping was done by six arrays through multiple sub-studies and was imputed based on HRC reference panel.[77] GWAS for 329 metabolites (N ranges from 971 to 8054, median N=6,610) was conducted within each genotype platform,[73] using the RVTESTS tool.[78] Each metabolite was first adjusted for age, fasting status, cohort, indicators of sub-studies for genotyping, indicators of sub-studies and case-control status within each sub-study for metabolomic profiling, and the first 4 genetic principal components (PCs); then the residuals were inversely normal transformed and regressed on the genotypes. Summary statistics from each platform were meta-analyzed using an inverse variance-weighted method using METAL.[73] In SOL, genotyping was performed using a customized Illumina Beadchip, and was imputed using 1000 Genomes Project phase 3 worldwide reference panel.[79] GWAS of metabolites were conducted in 3,933 SOL participants.[74] Residuals of each metabolite were first obtained by regressing on age, self-reported sex, eGFR, recruitment center, the first 5 genetic PCs, and origin; then the residuals were inverse normal transformed for genetic analyses adjusting the same covariates and 3 random effects terms (to account for genetic relatedness and environmental correlations) using a linear mixed-effect model in GMMAT with an additive genetic effect.[74] In ARIC, genotyping was performed using the Affymetrix 6.0 array and genome-wide imputation was performed based on 1000 Genomes Project Phase 3 reference panel.[80] GWAS of metabolites were conducted in 1772 African American and 1509 non-Hispanic White participants separately. Residuals of serum metabolites were first acquired after adjusting for age, sex, center, and eGFR; and then inverse-normal transformed residuals were used in genetic analysis, adjusting for the same covariates and the first 3 genetic PCs using liner regression models.[74] In WHI, genotyping of 1,256 women with metabolomics data was performed by 3 ancillary studies (Genomics and Randomized Trials Network, n=843; SNP Health Association Resource, n=216; Memory Study, n=198) using various Illumina or Affymetrix arrays[81] and the genetic data were imputed based on 1000 Genomes Project phase 3 version 5. GWAS of metabolites were conducted separately in each sub-study (race-specific) and were meta-analyzed using an inverse variance-weighted method using METAL.[73] Each metabolite was first inverse-normal transformed and adjusted for age, BMI, CHD case-control status in metabolomic profiling, and the first 10 genetic PCs; then the residuals from the linear model were further inverse-normal transformed for GWAS analysis using a linear regression implemented in RVTESTS[78] assuming an additive genetic effect. In CHS, genotyping was performed using the Illumina 370CNV or the HumanOmni1-Quad_v1 BeadChips and were imputed based on HRC r1.1 panel. Levels of each metabolite were first regressed on age, sex, fasting status, CHS clinic and 2 genetic PCs and then the model residuals were used for GWAS

implemented in RVTEST. Finally, GWAS of metabolites in 1,802 participants FHS were published previously, and we acquired the publicly available GWAS summary statistics.[76] Briefly, genotyping was performed using an Affymetrix 500K and a 50K Human Gene Focused Panel, followed by imputation of 2.5 million genetic variants by MACH based on the HapMap CEU population release 22. GWAS was conducted on normalized residuals of metabolites after adjusting for age and sex, using linear mixed effects models implemented by the lmekin function from the kinship R package, assuming an additive genetic effect. Population stratification was accounted for by adjusting for the genetic PC1 if $p<0.0001$.[76]

GWA meta-analysis for metabolites

GWAS summary statistics from each cohort were lifted over to Genome Build 37 and filtered, retaining SNPs with a minor allele frequency (MAF) ≥0.01 and imputation ratio ≥0.3. An inverse variance weighted fixed effect meta-analysis, implemented in METAL,[82] were used to combine GWAS results from the NHS/HPFS, SOL, ARIC, WHI, FHS, and CHS, for each metabolite based on their availability in each cohort. Genomic control was implemented before meta-analysis in each study and after meta-analysis.[82] Of the original 469 metabolites included in the longitudinal analyses, the final GWAS were available for 458 harmonized metabolites with the total sample size ranging from 1,074 to 18,590 (median n=8,611). We compared significant mQTLs identified at $P<5\times10^{-8}$ and 1.09×10^{-10} (i.e., $5\times10^{-8}/458$) levels. Manhattan plots were derived using R package *CMplot*, and regional plots were draw with *LocusZoom*.[83] In a secondary analysis, we compared genetic effect heterogeneity between racial/ethnical groups at the identified mQTLs for T2D-associated metabolites. In this comparison, GWAS for non-Hispanic White individuals were from meta-analysis of NHS/HPFS, FHS, CHS, and White participants from ARIC and/or WHI (based on metabolites' availability in each cohort); for African American individuals, included Black participants from ARIC and/or WHI; and for Hispanic/Latino individuals, included only GWAS summary statistics from SOL.

To annotate whether the mQTLs we identified for the 165 T2D-associated metabolites at $P<1.09\times10^{-10}$ are novel or have been previously reported, we compared our mQTLs to those reported in 8 major prior studies that had a sample size of ≥4,000 (i.e., with sufficient power) and applied liquid chromatography-mass spectrometry LC-MS to assess metabolites (i.e., the same methods used in our cohorts).[84-91] We matched the reported metabolites to ours primarily by HMDB ID and then by name. We considered a locus for a metabolite as "previously reported" if, for the same metabolite, the reported lead genetic variant was 1) the same lead variant as in our study; or 2) not the lead variant but was significant in our study; or 3) not in our study but within the clumping range of our identified locus. We considered a locus for a metabolite as "novel" if, our locus was not reported previously for this metabolite, or this metabolite was not previously reported in these studies.

Lead variants for metabolites, pathway analysis and proportion of variance explained

We used the PLINK *clumping* function to identify independent genetic variants associated with each metabolite at the genome-wide significant level ($P<5\times10^{-8}$ and $r^2<0.01$ in a 1000kbp window). If no variant showed $P<5\times10^{-8}$ for a metabolite, a single lead variant with the smallest *P* value was used. The use of this criteria (instead of $P<1.09\times10^{-10}$) was to identify independent

lead variants associated with each metabolite and to allow our analyses to include all metabolites for unbiased result comparison between T2D-associated *vs.* non-associated metabolites. Gene annotation of top variants was conducted using the SNPNexus web tool.[92] We conducted canonical pathway enrichment analyses using the MetaCore (Clarivate, PA, USA) with the default background.[93] Two separate analyses were conducted – one used genes annotated to mQTLs of T2D-related metabolites and the other used genes annotated to mQTLs of non-associated metabolites; and we then compared results for all and for top pathways between these two sets of enrichment analyses. Given the biochemical nature of our metabolites, most of them are not polygenetic traits; as such, we did not use Linkage Disequilibrium Score Regression (LDSC) to estimate h^2. Instead, for each metabolite, we calculated its R^2 explained by independent lead genetic variants identified from aforementioned clumping function, using the formula $\sum_{i=1}^{k} \beta \times \beta \times 2 \times \mathrm{MAF} \times (1 - \mathrm{MAF})$, with k being the numbers of independent lead variants, β being the association coefficient between the variant and the metabolite, and MAF being the minor allele frequency of the variant. We compared the R^2 distribution for the T2D-associated metabolites to that of non-associated metabolites using Wilcoxon test.

Genetic correlation between metabolites and clinical traits reflecting T2D pathophysiology

We curated GWAS summary statistics for T2D from the DIAGRAM consortium multi-ancestry meta-analysis (180,834 T2D cases and 1,159,055 controls).[94] We acquired GWAS summary statistics for fasting insulin (N=98,210);[95] proinsulin (N= 45,861);[96] HOMA-IR and HOMA-B (N= 51,750);[97] and insulin sensitivity index (ISI, N=53,657) and insulin fold-change (IFC; N=55,124) during oral glucose tolerance test after adjusting for BMI,[98] from meta-analyses of the MAGIC consortium. We acquired summary statistics for BMI and waist-hip ratio (N ~700,000) from the GIANT consortium meta-analysis;[99] and summary data for clinical lipid biomarkers (N~1,500,000) from the Global Lipids Genetics Consortium.[100] In addition, we conducted GWAS for HBA1c (N= 390,982), subcutaneous fat volume (N= 37,912), visceral fat volume (N= 37,912), liver proton density fat fraction (PDFF; N=29,512), pancreas PDFF (N= 28,624), and liver enzymes (N~ 390,000) in the UK Biobank. Inverse normal transformed clinical traits were regressed on genotypes using linear mixed regressions implemented in BOLT-LMM, considering inter-individual relatedness and adjusting for age, age^2, sex, study center, and the first 20 genetic PCs.

We examined genetic correlations between circulating metabolites and the aforementioned clinical traits using LDSC based on their GWAS summary statistics overlapping with the 1.2 million HapMap3 variants after excluding the major histocompatibility complex (MHC) region in the European population.[101] We compared the distribution of genetic correlations with each trait for the T2D-associated *vs.* non-associated metabolites, using chi-squared test, and *FDR*<0.05 (correcting for comparisons tested) were considered as statistically significant.

Genetic colocalization analyses

Between mQTLs and tissue-specific eQTL: We obtained tissue-specific cis-eQTLs summary statistics from the GTEx portal (GTEx v8) and focused our analyses on 47 tissue types collected from human body, excluding cultured cell types.[102,103] First, we examined the shared causal

variants between each circulating metabolite with tissue-specific transcriptome in each of the 47 tissue types using colocalization analysis implemented in the coloc.abf() function in R package 'coloc' v5.[104] The inputs were the GWAS summary statistics (e.g., marginal effect size, standard errors and MAF) for genetic variants within ±500 kb of the independent lead variants (P $<5\times10^{-8}$; if no variant showed $P<5\times10^{-8}$ for a metabolite, the single lead variant with the smallest P value was used) for each metabolite. The posterior probability of H4 (PPH4) informs the posterior probability of a shared causal variant between a circulating metabolite and a cis-gene expression in a particular tissue type; and we used PPH4 >0.8 as strong evidence of colocalization. Within each tissue type, we then tested whether the proportions of metabolites showing significant colocalization with tissue-specific gene expressions are higher among the T2D-associated *vs.* non-associated metabolites, using univariant logistic regression; and a one-sided *FDR*<0.05 (correcting for 47 tissue types) was considered as statistically significant.

Between *mQTLs and GWAS of T2D:* To ensure statistical power, we obtained GWAS summary statistics for T2D from the DIAGRAM consortium multi-ancestry meta-analysis (180,834 T2D cases and 1,159,055 controls).[94] Genetic colocalization analyses between T2D-associated metabolites and T2D were conducted using the same *coloc* methods, and within the same ranges on mQTLs as conducted for mQTL-eQTL colocalizations. We used PPH4>0.8 as strong evidence of colocalization between a circulating metabolite and T2D. We further aligned significant mQTL-T2D colocalizations with tissue-specific eQTL-mQTL colocalizations by metabolites and shared causal variants, to help interpret the functionality of the metabolites in T2D pathogenesis.

Mendelian randomization (MR) analysis of T2D-associated metabolites and T2D risk

We conducted MR analyses between each of the 233 T2D-associated metabolites and T2D risk. GWAS summary statistics for T2D were acquired from DIAGRAM consortium.[94] We applied 4 MR methods implemented in the *MendelianRandomization* R package,[105] acknowledging that each method has strengths and limitations. We used the MBE (mode-based estimate) as the main method, as it is generally conservative, less likely to have false positive findings, and robust to outliers.[106] We additionally applied the weighted-median (robust to outliers but less efficient), inverse-variance weighted (IVW; powerful but sensitive to IV invalidity), and MR-egger (sensitive to outliers and IV strength) methods[106] to help indicate result consistency. While IVW can work with a single variant through Wald test, the other 3 methods require 2-3 variants as the instrument variables (IVs). When testing the direction from metabolites to T2D risk, we used the following IV selection strategy to ensure all 4 MR methods could run on the same IVs: we removed the *HLA* region (hg19 chr6 29M-34Mbp); we then first acquired independent variants using *clumping* ($P<5\times10^{-8}$ and r^2 <0.01 in a 1000kbp window); if less than 3 variants were identified, we reduced the clumping P threshold (to 1×10^{-7}, 1×10^{-6}, etc.) until at least 3 independent variants could be identified. MR-egger intercept was extracted to indicate IV validity. We defined a significant causal relationship as *FDR* from MBE <0.05 (correcting for 233 metabolites) with at least two other MR methods showing the same effect directions as those from MBE.

We conducted two sensitivity MR analyses testing direction from metabolites to T2D risk. First, considering that some loci were associated with multiple metabolites, we removed any variants mapped to the top 3 recurrent loci from the IVs of metabolites, including *GCKR* (±

500kbp from top lead variant), *ZNF259* (± 500kbp), and the *FADS* cluster (± 1Mbp), and repeated the IVW analyses. Second, we repeated the IVW analyses using only independent variants clumped at $P<1.09\times10^{-10}$ (i.e., $P<5\times10^{-8}/458$ metabolites). To examine potential reverse causation, we also tested the direction from T2D to metabolites, using independent lead variants associated with T2D at $P<5\times10^{-8}$ as IVs.

In a secondary two-sample MR analysis, we tested the direction from BMI to each metabolite, specifically for the 148 metabolites identified as potential mediators between BMI and T2D risk from our longitudinal mediation analysis. GWAS summary statistics for BMI were acquired from the GIANT consortium meta-analysis (N ~700,000).[99]

Metabolome-wide association analysis for modifiable risk factors of T2D

To examine the association between each metabolite and baseline risk factors, inverse normal transformed metabolite levels were regressed on age, sex (only in SOL), current smoking status, BMI, physical activity, intakes of 15 commonly consumed food groups, and fasting status simultaneously, together with cohort-specific covariates. In the model, BMI and physical activity levels were natural log-transformed and standardized; food intakes were unified in unit of servings/day with the higher end winsorized (with cohort-specific cutoffs) to remove outliers. All analyses were conducted separately in NHS/HPFS (combining all three cohorts with additional stratification by cohorts, sub-studies, and nested case-control studies within sub-studies), SOL, and WHI (among non-Hispanic White individuals and individuals of other races and ethnicities, separately). Association coefficients between metabolites and each particular risk factor were then combined across cohorts and analytical sets using a fixed-effect, inverse-variance weighted meta-analysis. To acquire R^2 of each metabolite explained by specific risk factors, we first calculated the R^2 in each analytical set using the formula $\beta \times \beta \times variance(\text{risk factor})/variance(\text{metabolite})$, with the β being the association coefficients between the metabolite and the risk factor; we then calculated the averaged R^2 across all analytical sets. We compared the distributions of R^2 for T2D-associated metabolites *vs.* other metabolites using the Wilcoxon test.

Mediation analysis between risk factors, metabolites, and T2D risk

Within NHS/HPFS, SOL, and WHI, 4 baseline risk factors (namely, BMI, physical activity, coffee/tea consumption, and red/processed meat intake) showed consistent prospective associations with T2D risk that are aligned with cumulative epidemiological evidence. We then conducted mediation analyses to identify metabolites significantly mediated (and if yes, to what extent) the associations between each of these risk factors and T2D risk, using the R package CMAverse.[107] Metabolites considered for mediation analysis were 1) associated with T2D risk in Model 2 of meta-analysis at FDR<0.05; 2) showed independent associations with the baseline risk factor (after mutually adjusted for other risk factors) in meta-analysis combining NHS/HPFS, SOL, and WHI at FDR<0.05; 3) associations directions with the risk factor and T2D risk were consistent with the pre-assumed epidemiological relationships between the risk factor and T2D risk. Analyses were conducted separately in NHS/HPFS, SOL, and WHI (by race), adjusting age, sex, smoking, BMI, and physical activity (if not included as the tested risk factor), total calorie intake, and other cohort-specific covariates. The mediation analysis produced three association paths, total effect (the overall association effect between a risk factor and T2D risk),

indirect effect (the association effect between the risk factor and T2D risk that was mediated by the metabolite), and direct effect (the remaining association effect between the risk factor and T2D risk). We combined total, indirect, and direct effects from each analytical set using a fixed effect meta-analysis, respectively. A metabolite showing significant indirect effect at $FDR<0.05$ (correcting for metabolites tested) and a consistent effect direction between the indirect effect and total effect, was considered as a potential mediator between this particular risk factor and T2D. The mediated proportion of the association was calculated as the ratio of the indirect effect to the total effect.

A multi-metabolite signature for incident T2D prediction

To develop a metabolomic signature for T2D risk prediction, we focused on metabolites shared between the Broad Institute and the Metabolon platforms (excluding glucose) to increase the generalizability of our signature to future studies. For model development and testing, to avoid overfitting, we employed a leave-one cohort-out across-validation approach, in which we set aside one of the cohorts as the testing set each time, and trained a prediction model for the set-aside cohort using data from all other cohorts. Given that cohorts included in our study have different study designs, population structure and demographics, follow-up length, data collection instruments, and availability of metabolites, we were not able to pool individual level data from multiple cohorts together for model training. As such, we applied a two-step model training approach in which the prediction model was trained in one representable cohort but still leveraged the association data from multiple other cohorts. Among our study cohorts, WHI assessed the most metabolites from the Broad Institute platform and the most metabolites shared between the two platforms for all its participants, and was therefore selected for model training using penalized regression (which requires complete data for all participants). In each iteration (i.e., for each of the held-out testing cohorts), we first conducted a metabolome-wide meta-analysis for T2D risk of all the cohorts except WHI and the held-out cohort. Then, metabolites with significant association with T2D risk (FDR<0.05) in the first step and shared between the two metabolomic platforms, were used as input in a Cox regression with elastic net regularization, implemented using the *glmnet* R package,[108] to construct a metabolomic signature model for T2D prediction in WHI. The derived model from the elastic regression was further applied to the independent held-out cohort to calculate a metabolomic signature score. Within WHI, a leave-one-out cross-validation (LOOCV) approach was used to acquire an unbiased metabolomic signature score. Finally, we evaluated the performance of the metabolomic signature score for T2D risk prediction based on the model AUC in each held-out cohort (**Extended Data Fig. 8**).

We conducted two sensitivity analyses during model development. One was to use SOL, which measured the most metabolites from the Metabolon platform for all its participants, as the representative training cohort instead of WHI. This analysis showed similar results, though models trained using WHI had slightly better performance in more held-out testing cohorts *vs.* that of SOL (**Extended Data Fig. 8**). The other was to compare the model performance of elastic net *vs.* lasso regularizations[108] in WHI (and separately in SOL) based on LOOCV. The results showed that elastic net regression had a compatible but slightly better prediction for T2D risk *vs.* lasso regression, reassuring us to use elastic net regression in model development (**Supplemental Fig. S13**).

The metabolomic signature scores from the prior steps were standardized (z-score transformation or inverse normal transformation) before analyses. To evaluate whether the metabolomic signature improved the T2D risk prediction, we fitted three sets of logistic (in SOL, and T2D nested case-control sub-study in NHS) or Cox models (all other datasets). This included regressing incident T2D on the metabolomic signature only; on a list of conventional risk factors including age, sex, smoking, lipid-lowering medication use, anti-hypertensive medications, family history of diabetes, hypertension, dyslipidemia, and BMI; and on both the conventional risk factors and the metabolomic signature. We compared the AUC between the conventional model *vs.* the conventional plus metabolomic signature model. The AUC for Cox regression was calculated at the 50th follow-up time. In a secondary analysis, we further included blood glucose (from metabolomic assays) in the conventional model to evaluate if the metabolomic signatures demonstrated added value beyond this T2D diagnostic biomarker. NHS/HPFS was not included in this analysis given the blood samples had delayed processing.

To evaluate if the metabolomic signature could stratify healthy individuals based on their future T2D risk, we calculated the crude incident rate of T2D across deciles of the metabolomic signature score, separately in each cohort. We also fitted logistic or Cox regression models to analyze the relative risk of T2D, comparing higher *vs.* lowest deciles of the metabolomic signature, adjusting for the same covariates in the model 2 from the main analysis. Analyses were conducted in each cohort separately and results were combined using a meta-analysis. To examine associations between the metabolomic signature with baseline risk factors, we applied linear regression to regress the metabolomic signature score on age, sex (if appropriate), current smoking status, BMI, physical activity, intakes of 15 commonly consumed food groups, and fasting status simultaneously, together with cohort-specific covariates. The analysis was conducted separately in NHS/HPFS, SOL, and WHI, and results were combined using a meta-analysis. *FDR*<0.05 (corrected for 18 risk factors) was considered as statistically significant.

Separately from the leave-one cohort-out cross-validation, we presented a metabolomic signature model for future studies using data from all of our study cohorts. For this model, we first conducted a metabolome-wide meta-analysis for T2D risk in all cohorts except WHI, and then used significant metabolites (FDR<0.05) as input in a Cox regression with elastic net regularization for T2D prediction in WHI. The selected metabolites and their coefficients of this final model, and comparisons with all individual models applied to each cohort, are in **Supplemental Table S18a**, showing high consistency, albeit slight differences, across all prediction models.

Supplemental Materials

Cohort-specific Acknowledgement

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The Boston Puerto Rican Health Study was supported by NIH grants, including P01AG023394 and R01AG055948 from the National Institute on Aging (NIA), and P50HL105185 from the NHLBI. Metabolomics profiling was funded, in part, by K01DK107804 from the NIDDK.

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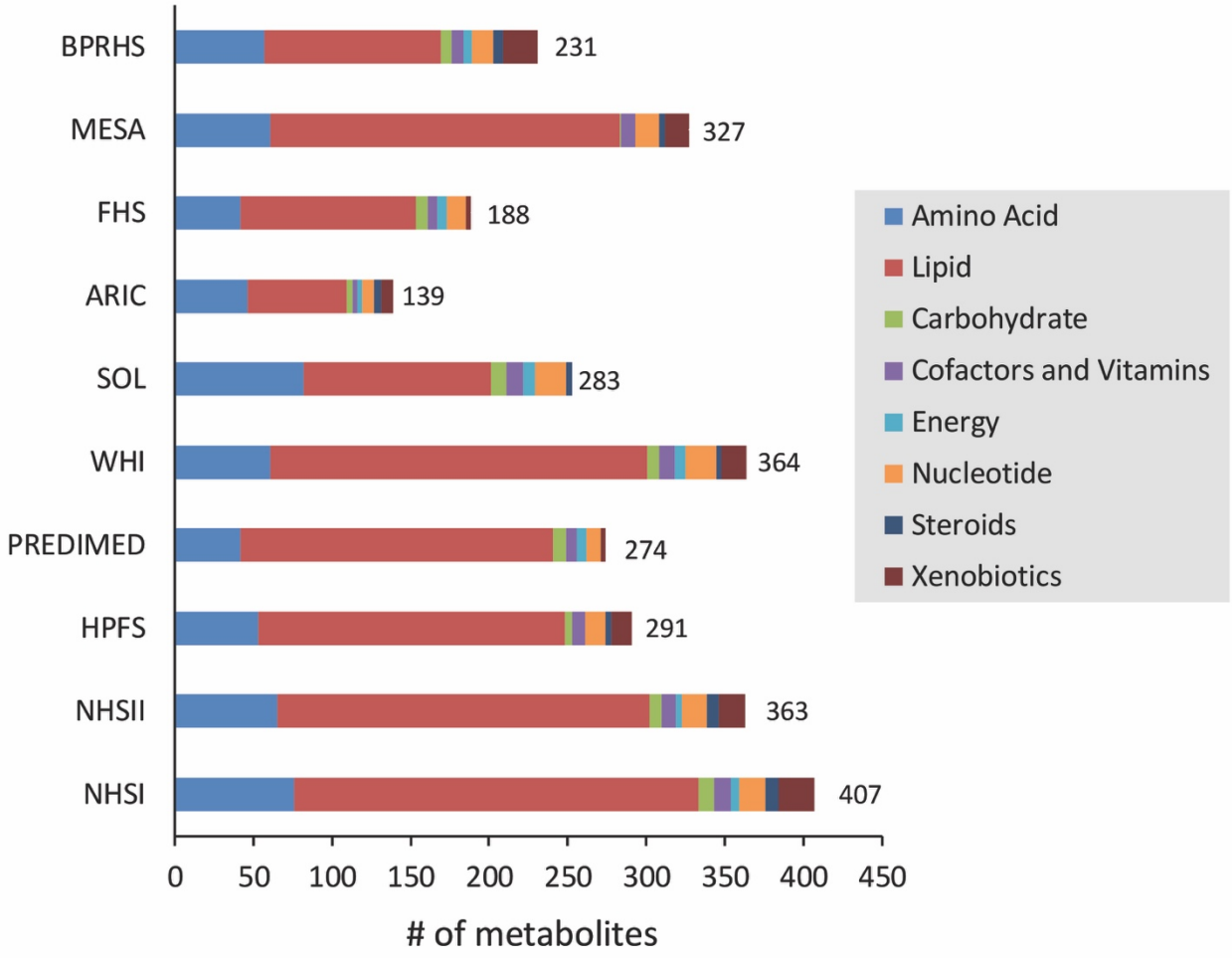
found at CHS-NHLBI.org. The provision of genotyping data was supported in part by the National Center for Advancing Translational Sciences, CTSI grant UL1TR001881, and the NIDDK Diabetes Research Center (DRC) grant DK063491 to the Southern California Diabetes Endocrinology Research Center. The study is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

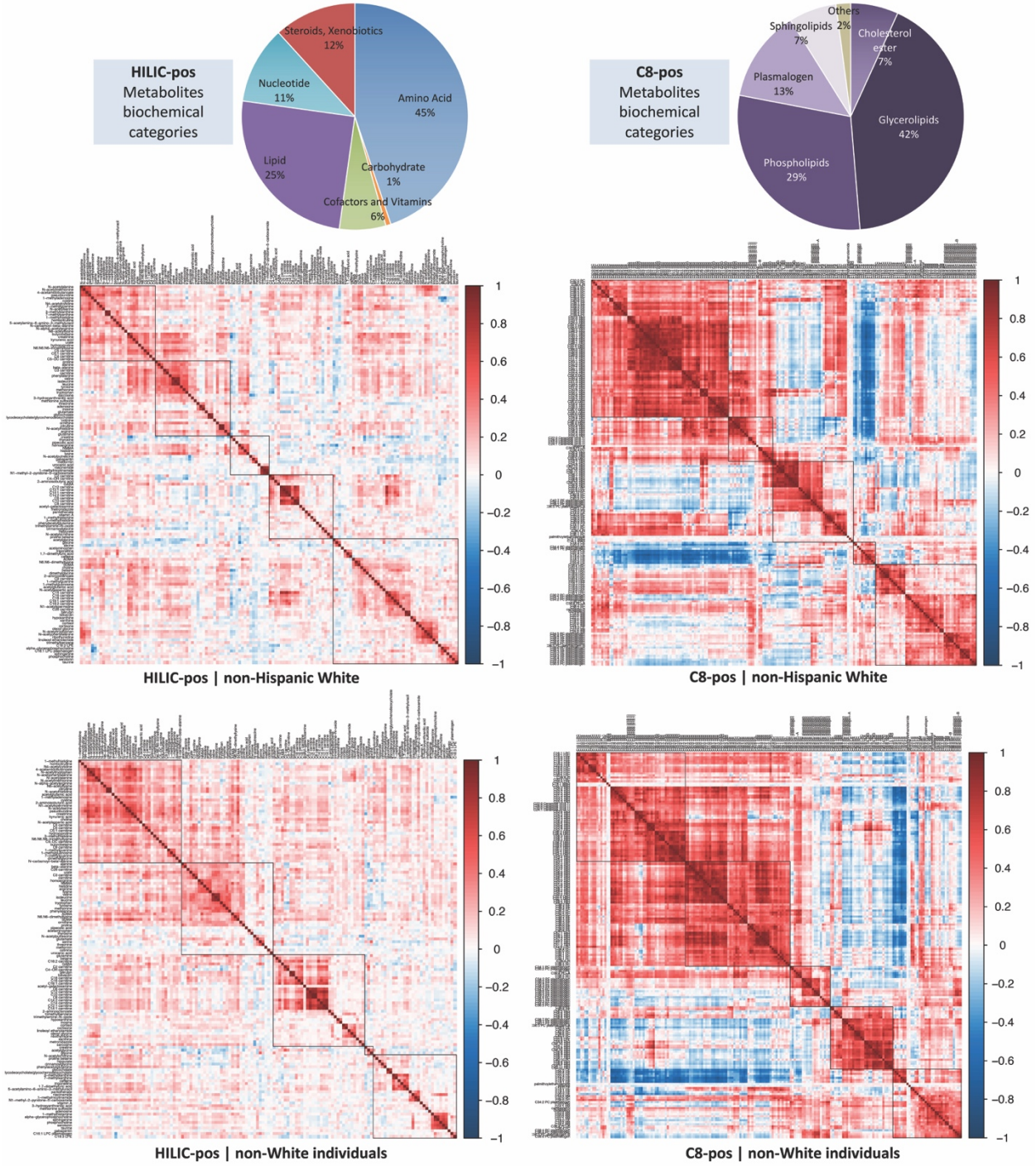
Supplementary Figures

Supplementary Figure S1. Harmonized metabolites in each of the 10 participating cohorts. We plotted the numbers of metabolites included in the prospective association analysis with T2D risk, colored by their biochemical category.

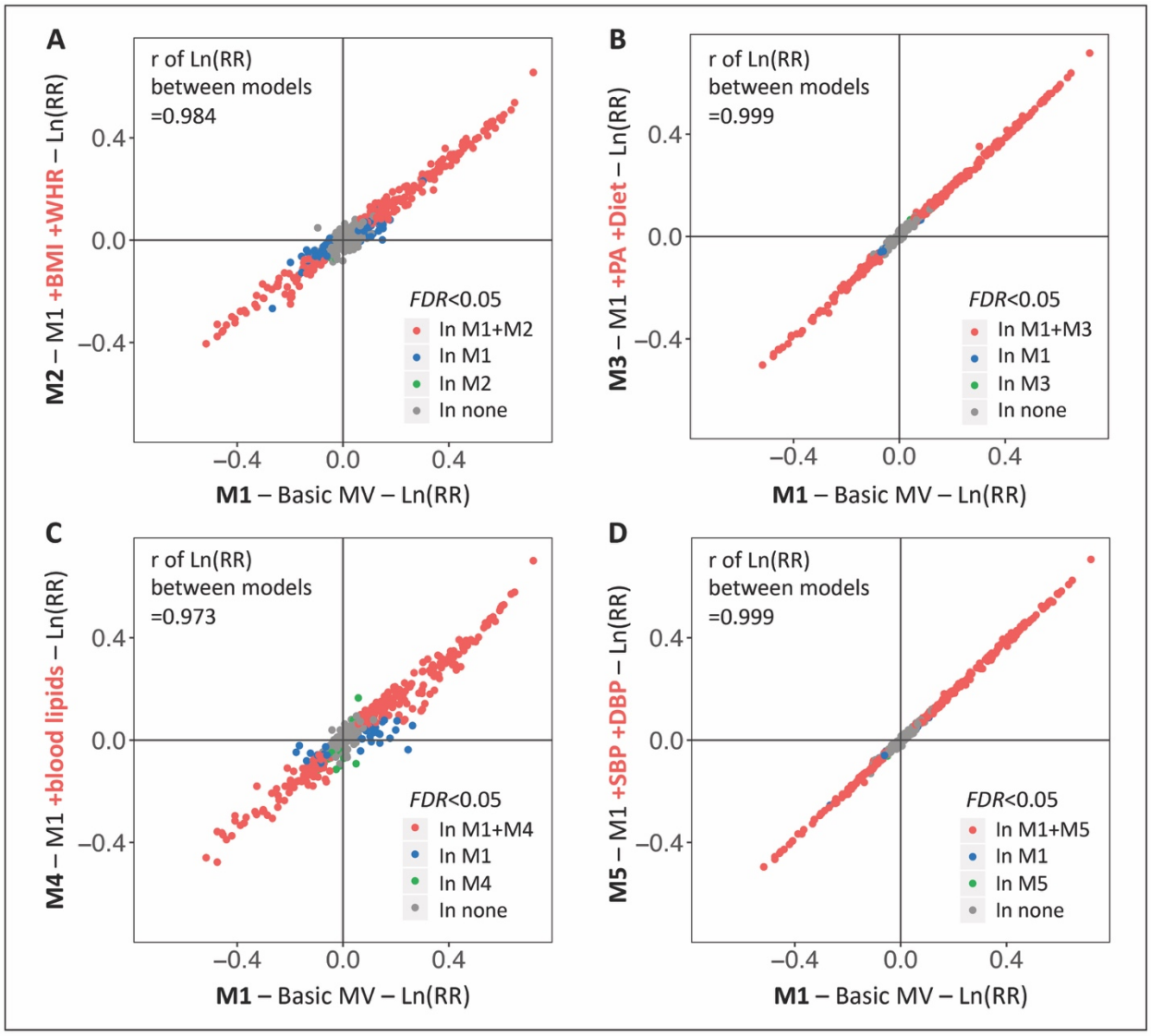


Supplemental Materials

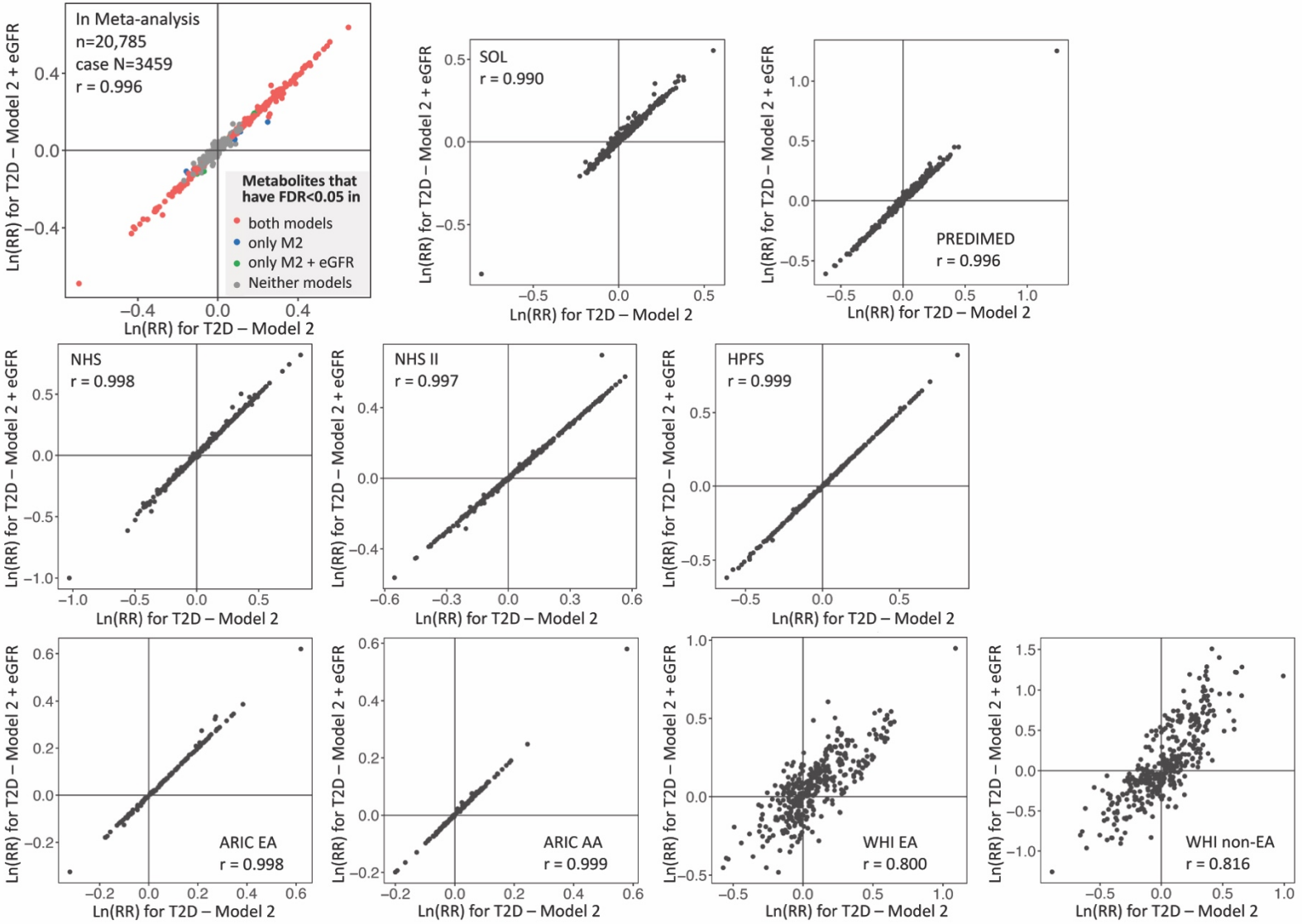
Supplementary Figure S2. Correlation matrices across metabolites. We used the Multi-Ethnic Study of Atherosclerosis as an example, as it included men and women with diverse racial/ethnic backgrounds and measured metabolites from various biochemical categories.



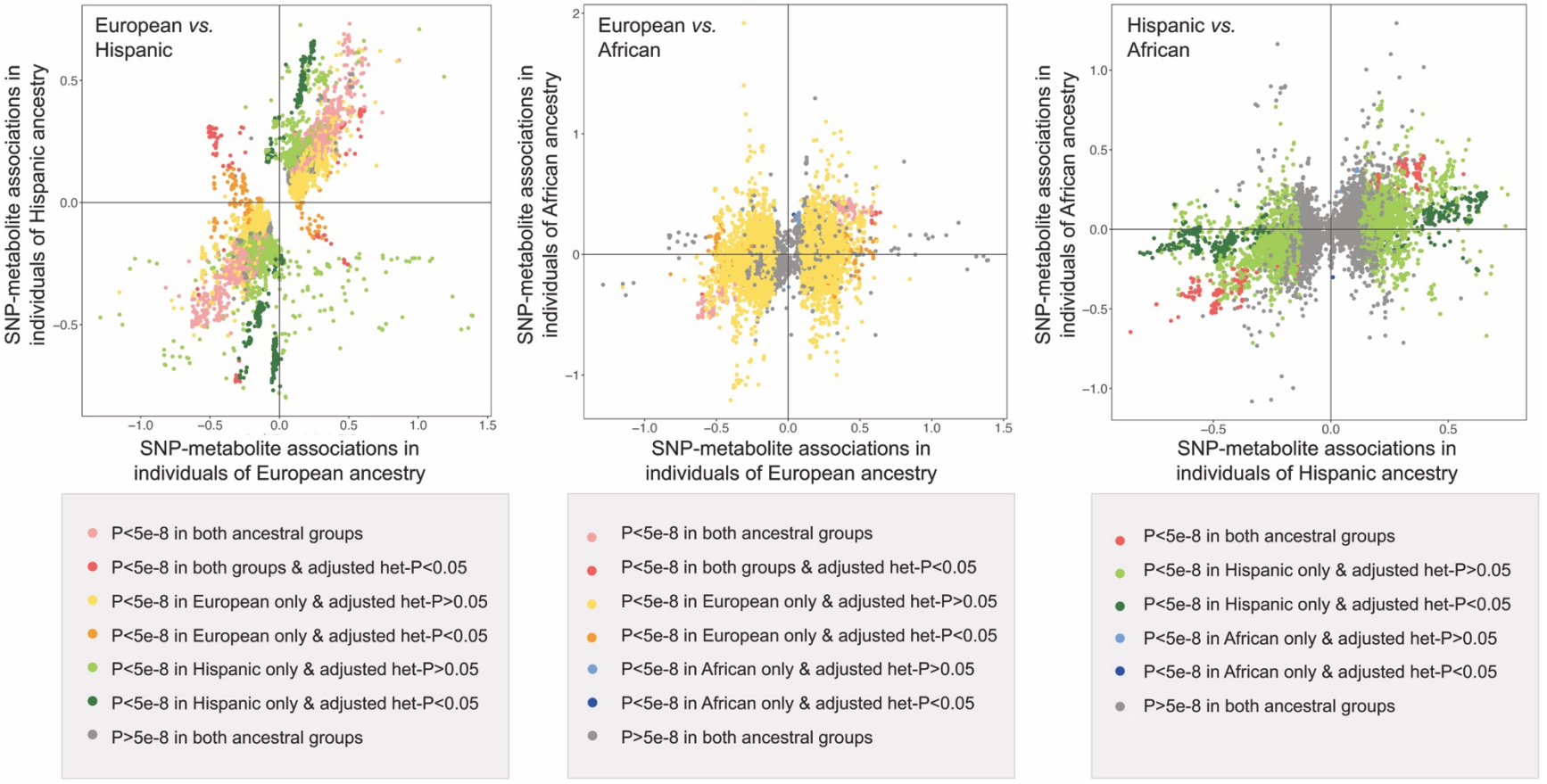
Supplementary Figure S3. Comparison of the associations between the 469 harmonized metabolites and T2D risk across 5 analytical models. In each cohort and stratified by major racial/ethnic groups, we applied cox regressions or logistic regressions to analyze associations between each metabolites (inverse normal transformed) and T2D risk. Results from all cohorts were combined using a meta-analysis. Both axis show natural log-transformed relative risk ratio (RR) for T2D risk per standard deviation increase in the levels of metabolites. The basic multivariable (MV) model (M1) was adjusted for age (years), sex, smoking (current/former/never), alcohol consumption (g/day), fasting status, lipid-lowering mediation use, anti-hypertensive medication use, hypertension, dyslipidemia, family history of T2D, and other cohort specific variables (see Supplementary Table S2). Based on Model 1, Model 2 (M2; the main model) further adjusted for body mass index (BMI) and waist-hip ratio (WHR); model 3 (M3) further adjusted for physical activity (METs h/week) and dietary quality; model 4 (M4) further adjusted for high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides; and model 5 (M5) further adjusted for systolic blood pressure (SBP) and diastolic blood pressure (DBP).



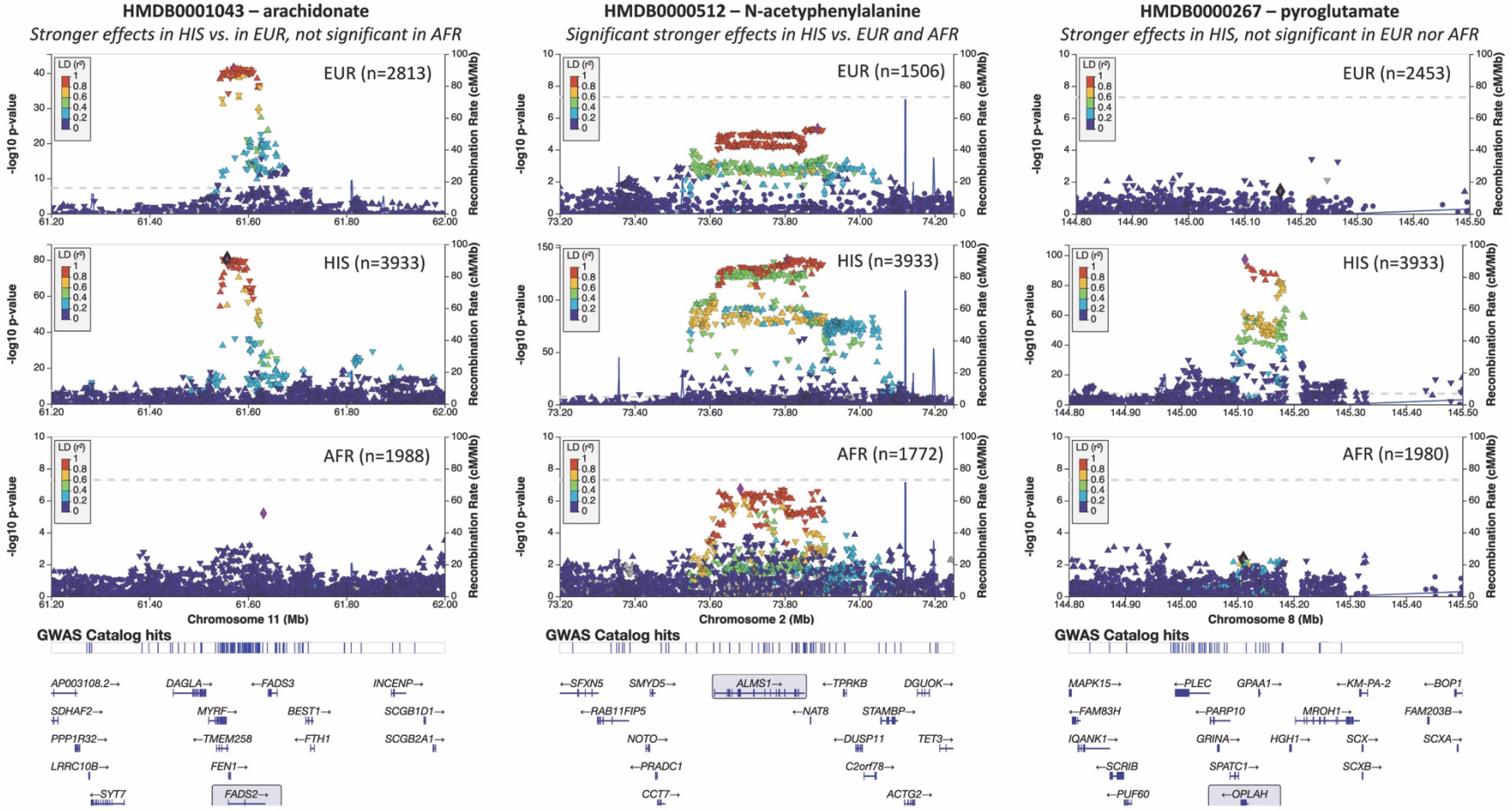
Supplementary Figure S4. Comparison of the associations between the 469 harmonized metabolites and T2D risk in main model *vs.* a sensitivity model further adjusting for eGFR. Main model is Model 2 described in Figure S3. Results were available in 7 cohorts. We presented the results from the meta-analysis and from each cohort by major racial and ethnic groups.



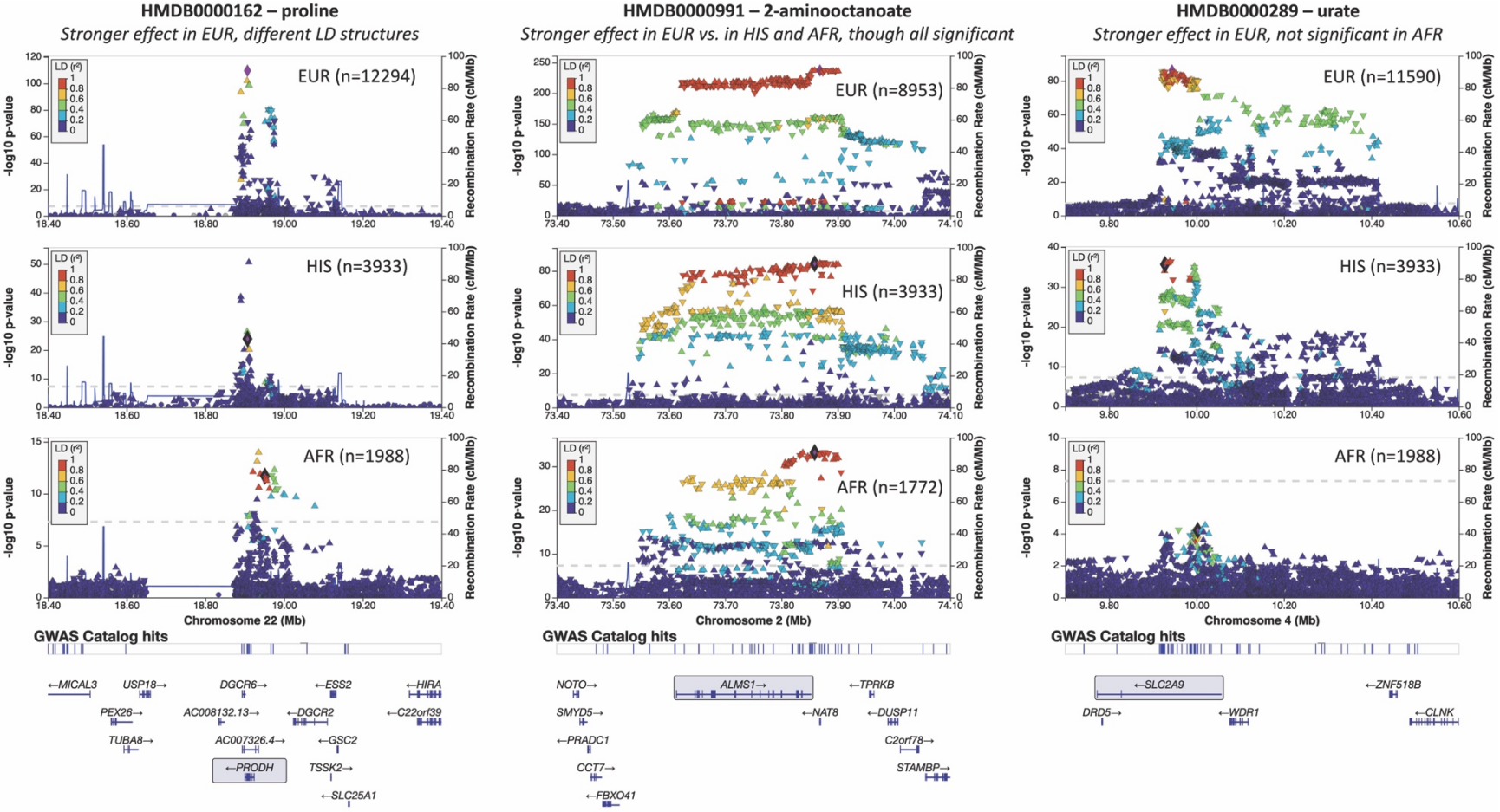
Supplementary Figure S5. Associations between genetic variants and metabolites at the identified mQTLs, comparing between racial/ethnic groups. Comparisons were focused on T2D-associated metabolites with mQTLs at $P<1.09\times10^{-10}$. We examined all metabolite-SNP pairs with $P<1.09\times10^{-10}$ from the overall meta-analysis; and 18,076 variant-metabolite associations (for 85 unique metabolites) were available for comparison between non-Hispanic White and Hispanic/Latino individuals, 23,649 (for 143 unique metabolites) were available for comparison between non-Hispanic White and African American individuals, and 15,828 (for 72 unique metabolites) were available for comparison between Hispanic/Latino and African American individuals. For each cross-ethnic comparison, *P-heterogeneity* values were corrected for multiple testing based on numbers of variant-metabolite associations tested.



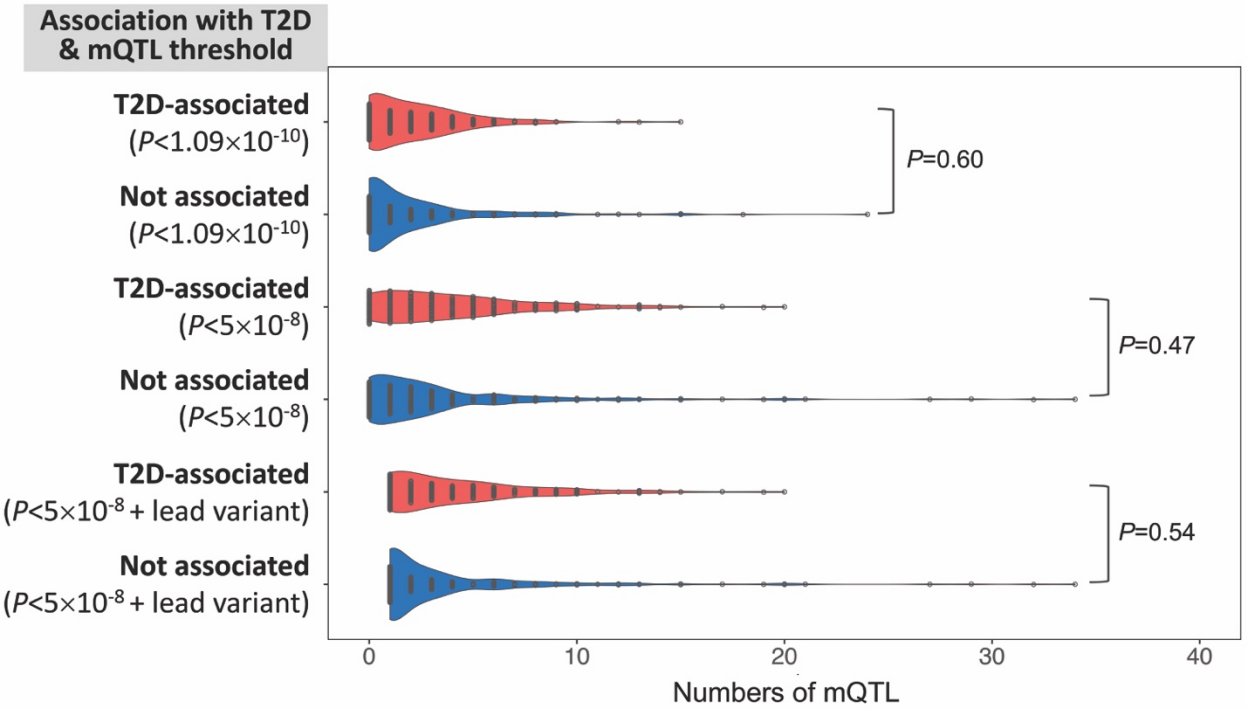
Supplementary Figure S6a. Comparison of mQTLs between racial/ethnic groups – example 1. Among the mQTLs identified in the overall sample, for variant-metabolite associations that showed significant between-ethnic effect heterogeneity (at the variant level, see Supplementary Figure S5), we then examined potential heterogeneity at the locus level. We observed two scenarios – below showcases the first scenario in which a locus was identified for a metabolite in one or two, but not all racial/ethnic groups.



Supplementary Figure S6b. Comparison of mQTLs between racial/ethnic groups – example 2. Among the mQTLs identified in the overall sample, for variant-metabolite associations that showed significant between-ethnic effect heterogeneity (at the variant level, see Supplementary Figure S5), we then examined potential heterogeneity at the locus level. We observed two scenarios – below showcases the second scenario in which a locus can be observed (significant, or as a non-significant signal) in all racial/ethnic groups but there is effect heterogeneity at individual variant level (i.e., lead variants were different), likely due to LD differences.

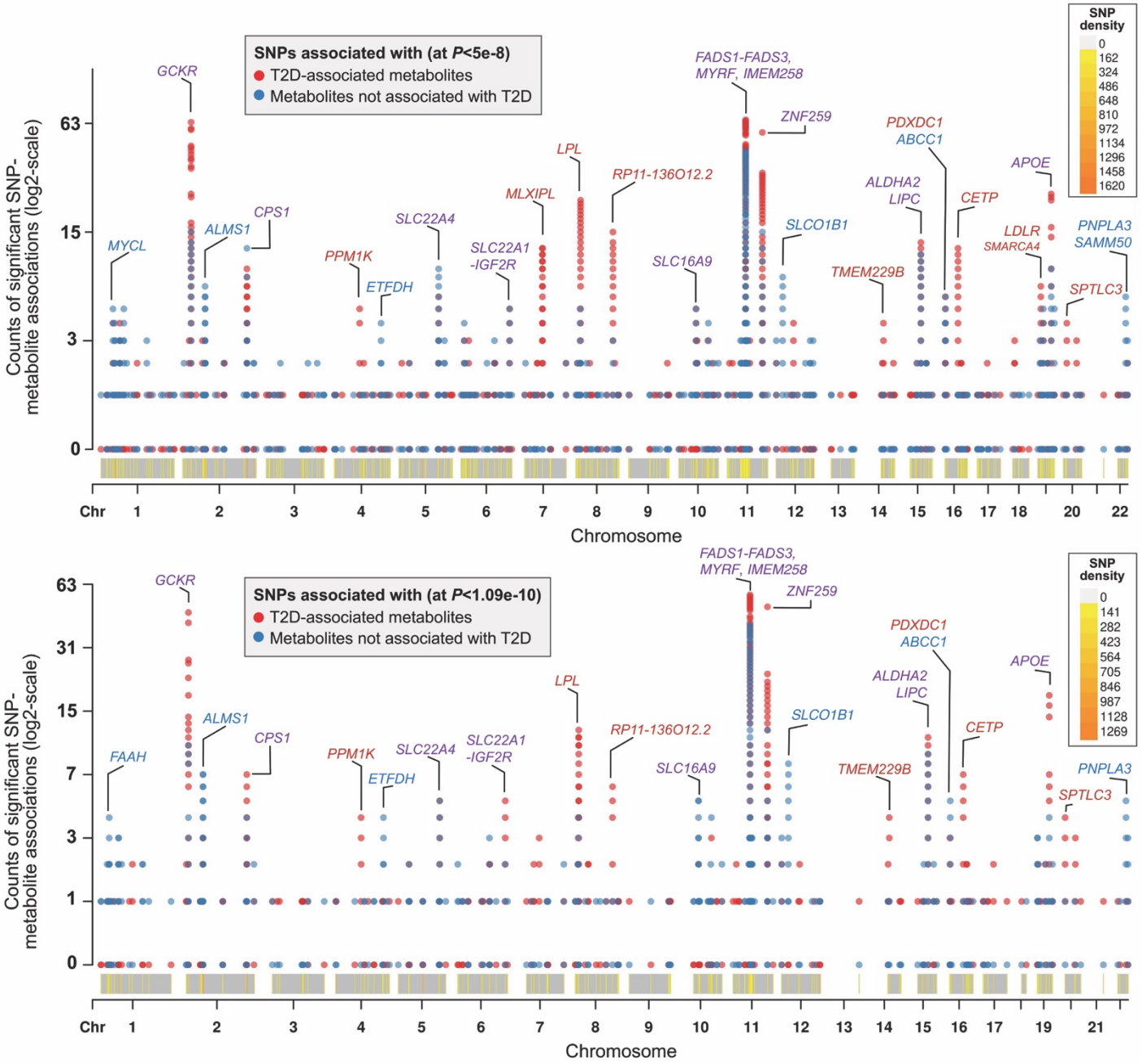


Supplementary Figure S7. Numbers of independent genetic variants per circulating metabolite, identified at different *P-value* threshold, comparing between T2D-associated metabolites *vs.* non-associated metabolites. We compared numbers of independent lead variants at (1) the genome-wide significance level with further correction for 458 metabolites ($P<1.09\times10^{-10}$); (2) the default genome-wide significance level ($P<5\times10^{-8}$); and (3) the genome-wide significance level of $P<5\times10^{-8}$ and, if a metabolite had no variant at $P<5\times10^{-8}$, further considering a single lead variant with the smallest P value.

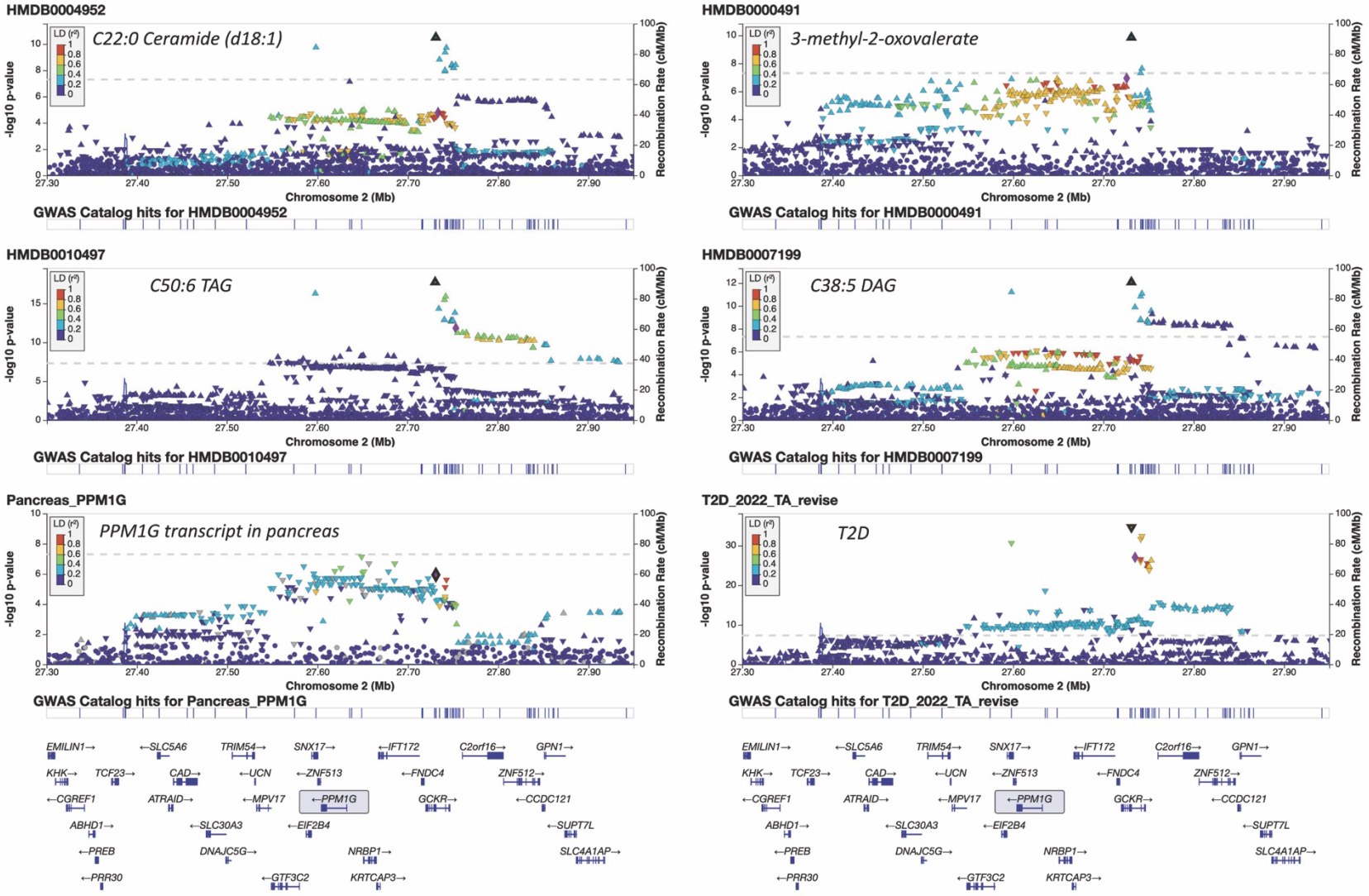


Supplemental Materials

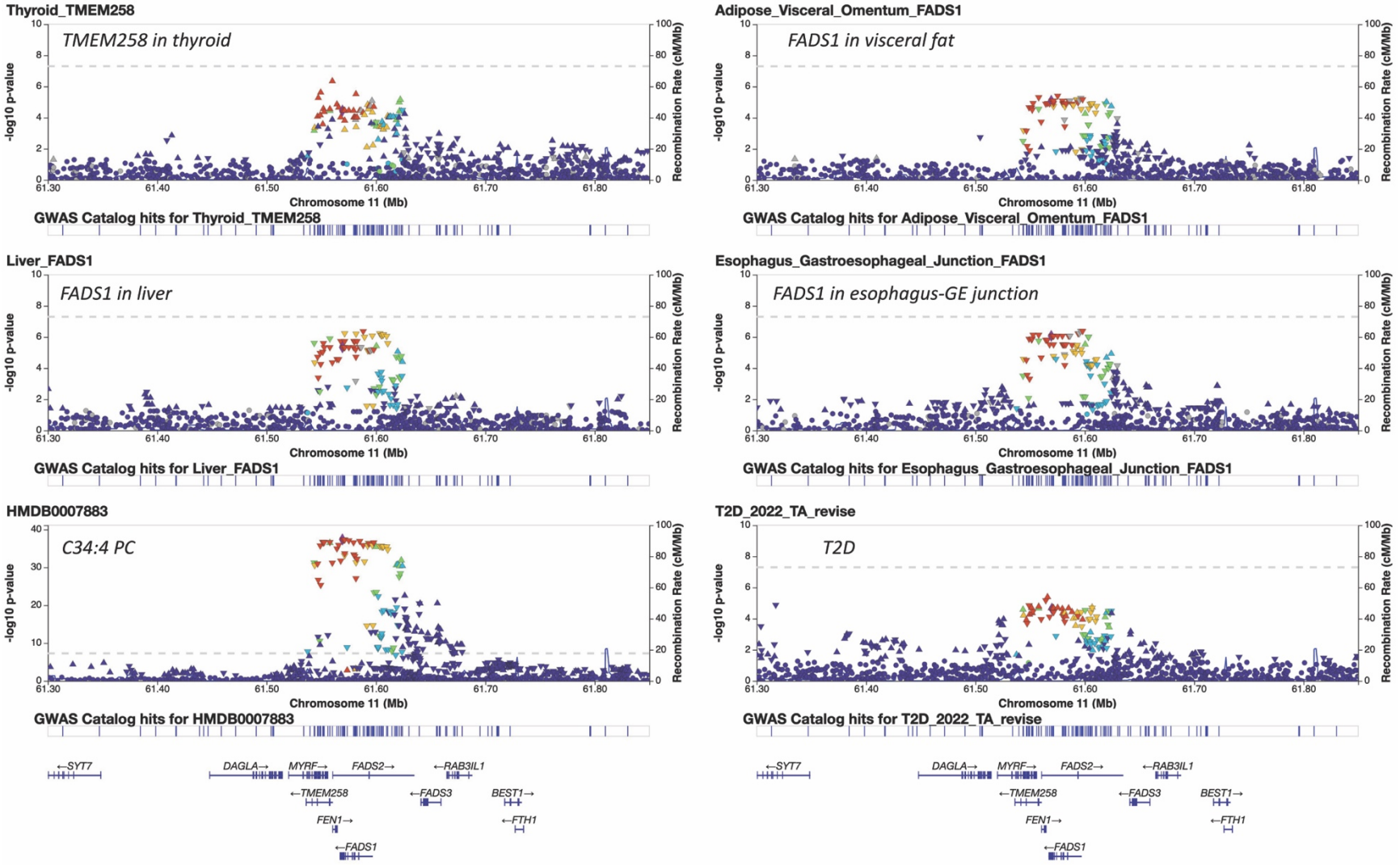
Supplementary Figure S8. Genetic determinants of circulating metabolites, stratified by metabolites' associations with incident T2D. The Manhattan-like plots show all significant genetic variants associated with any of our studied circulating metabolites, at the standard genome-wide significant level ($P<5\times10^{-8}$; upper figure) and after further Bonferroni corrections for 458 metabolites with genetic data ($P<1.09\times10^{-10}$; lower figure). The x-axis demonstrates chromosomal positions; y-axis shows the numbers of metabolites associated with each variants; and the color depicts the metabolites' association with T2D in our meta-analysis of prospective cohort studies. Genome-wide association study was conducted in each of the 8 cohorts by major racial/ethnic groups, and meta-analyzed using fixed effect meta-analysis in METAL. Among the 469 harmonized metabolites, 458 have GWAS summary data and were included in this figure.



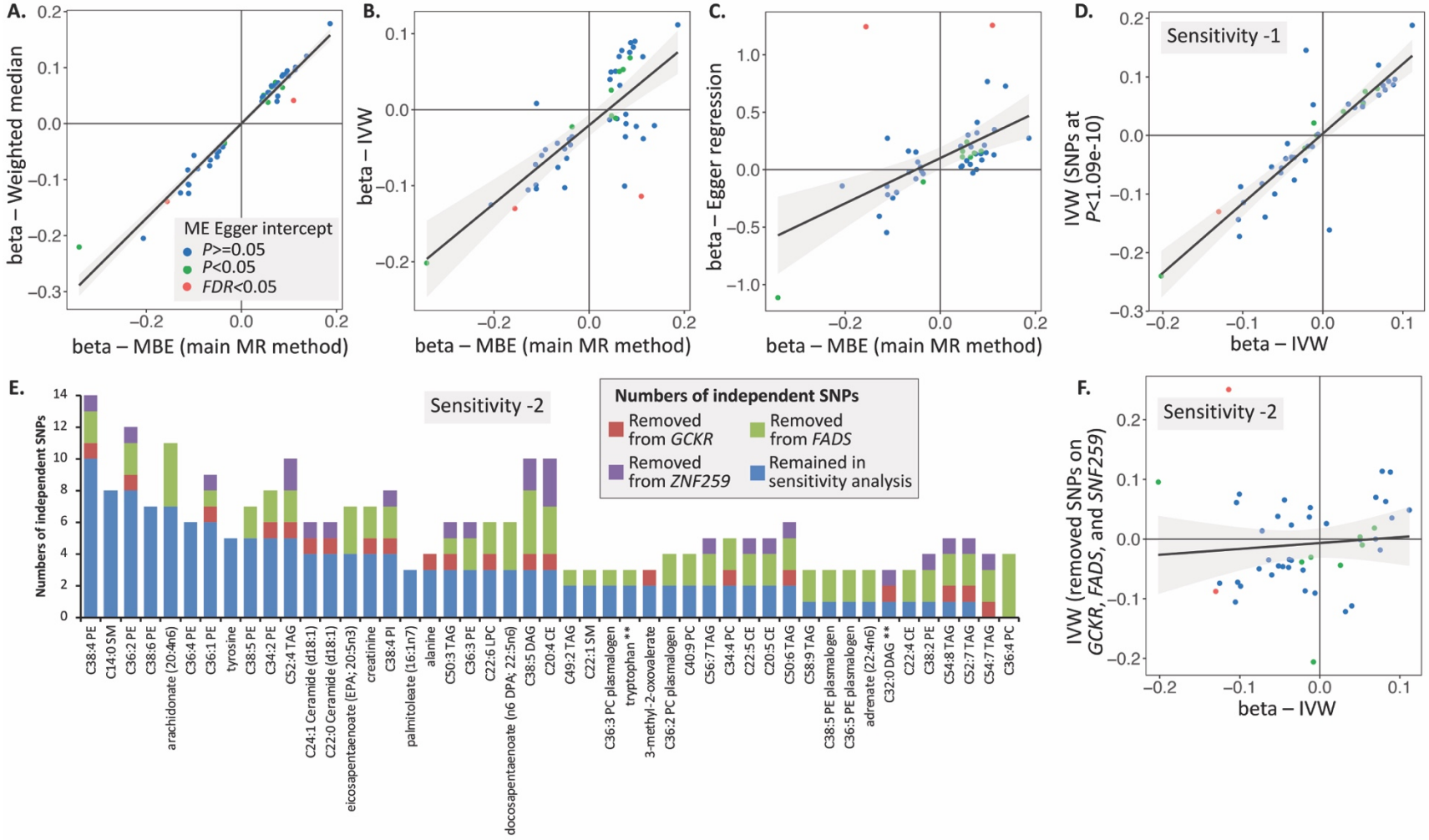
Supplementary Figure S9a. Genetic colocalization between tissue-specific gene expression, circulating metabolites, and T2D – example 1 at the *GCKR/PPM1G/IFT172* locus. The figure showcases the genetic colocalizations between circulating levels of C22:0 Ceramide (d18:1), 3-methyl-2-oxovalerate, C50:6 TAG, and C38:5 DAG (among other metabolites), *PPM1G* expressions in pancreas, and T2D (PPH4>0.8), with rs1260326 identified as the likely shared causal variant.



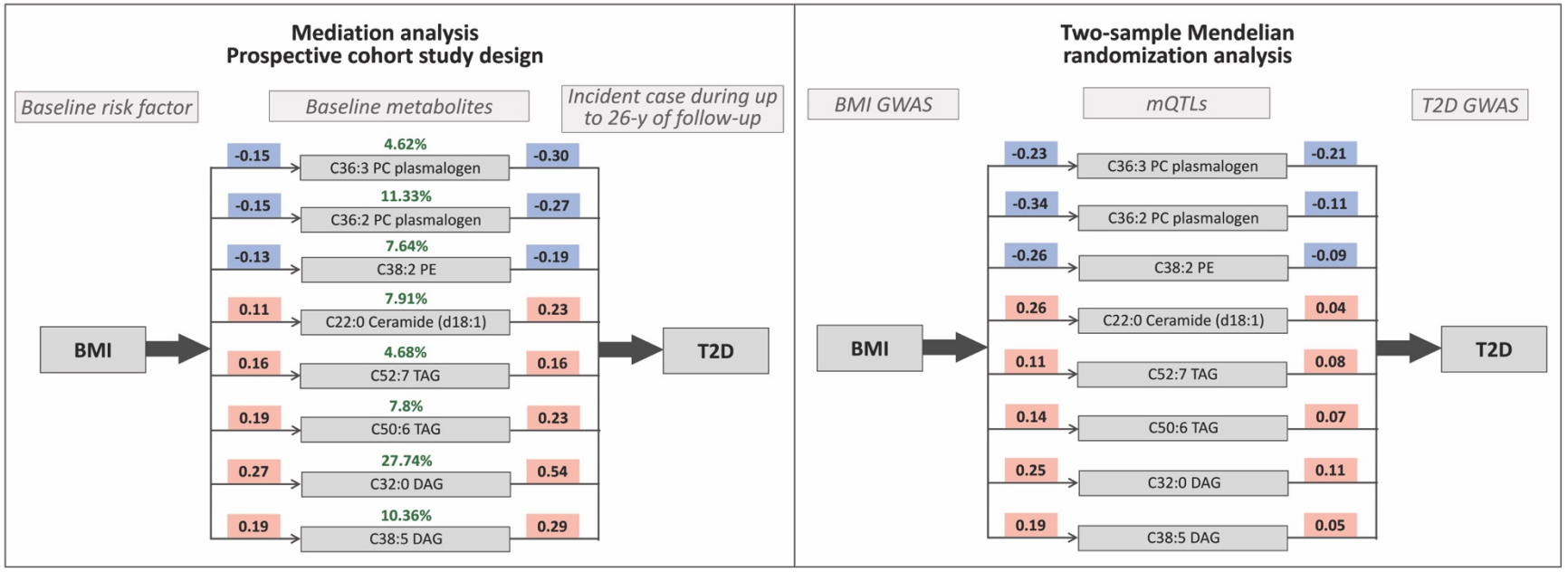
Supplementary Figure S9b. Genetic colocalization between tissue-specific gene expression, circulating metabolites, and T2D – example 2 at the *FADS1/TMEM258* locus. The figure showcases the genetic colocalizations between *TMEM258* expressions in thyroid, and *FADS1* expression in visceral fat, liver, and esophagus gastroesophageal junction, circulating C34:4 PC levels, and T2D (all PPH4>0.8), with rs174545 being the likely shared causal variant.



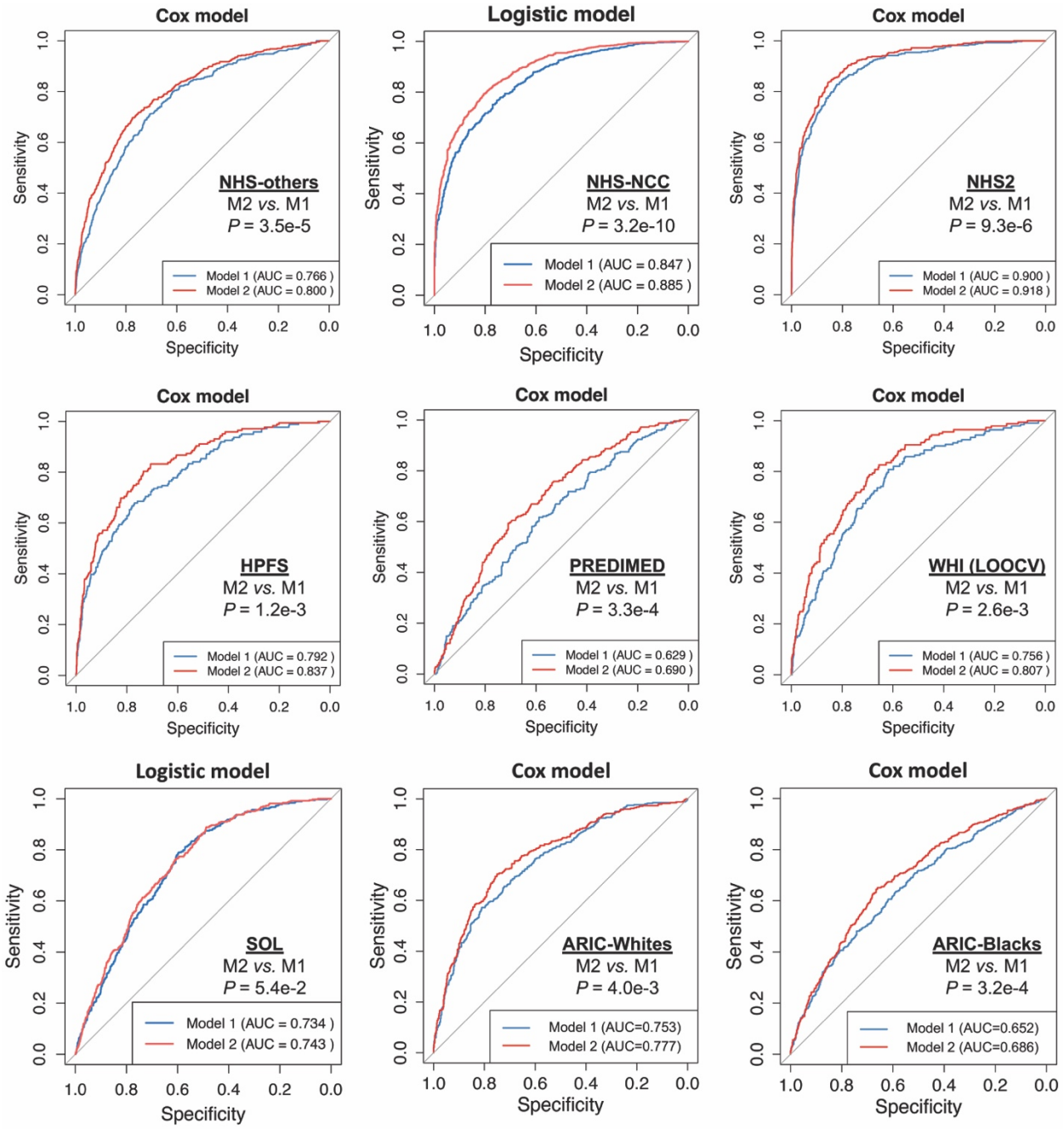
Supplementary Figure S10. MR sensitivity analyses for the 47 metabolites potentially causal for T2D. Of the 233 T2D-related metabolites with genetic data, we identified 47 showed *FDR*<0.05 in MBE and consistent effect directions from at least two other MR methods. **A-C**, comparisons of effect sizes across the 4 MR methods testing the direction from metabolite to T2D risk. **D**. comparisons of effect sizes from IVW MR when using primary instrument variables (IVs) *vs.* using the lead variant at $P<1.09\times10^{-10}$. In another sensitivity analysis, we removed variants on *GCKR*, *ZNF259*, or *FADS* from IVs and repeated the IVW analysis. **E** shows the numbers of variants removed for each metabolite; **F** compares the effect sizes between the primary analysis and this sensitivity analysis.



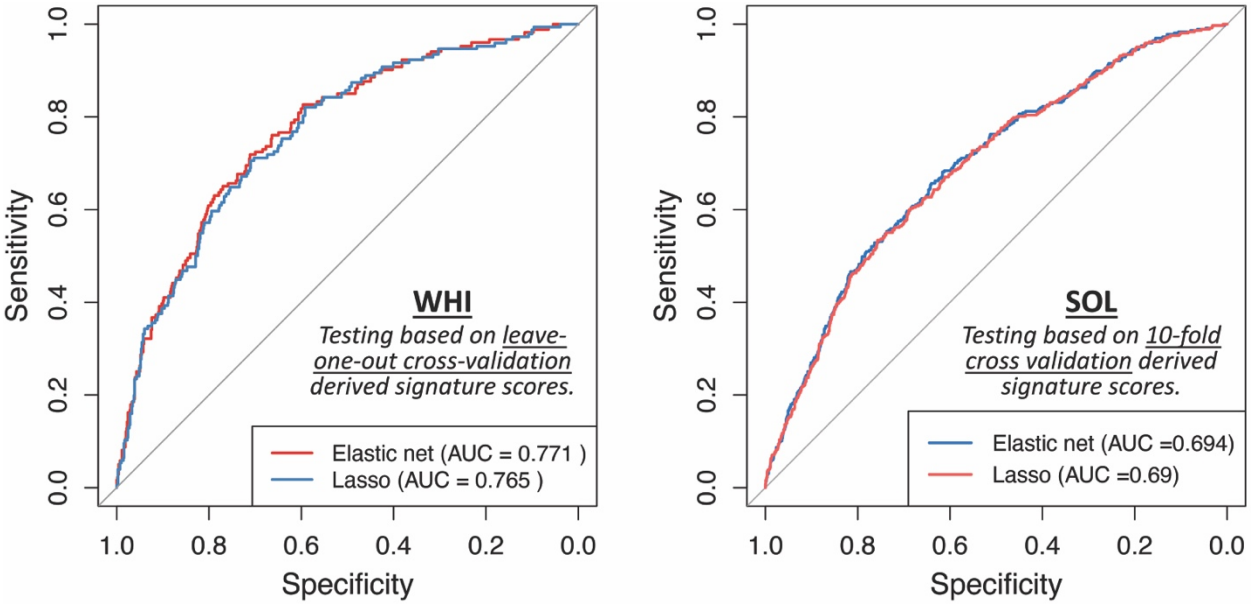
Supplementary Figure S11. Metabolites that likely mediated the association between BMI and T2D risk, supported by prospective cohort-based mediation analysis and two-sample Mendelian randomization analysis. Among the 148 metabolites identified as significant mediators between baseline BMI and T2D in prospective cohort analyses, we further incorporated results from MR analysis testing the direction from BMI to metabolites, and from metabolites to T2D. Relationships that further satisfied the following criteria are selected to present in here: 1) for the direction from metabolite to T2D, MR results are significant in MBE with consistent directions from at least 2 other MR methods; 2) for the direction from BMI to metabolite, nominally significant MR results consistently in at least 3 MR methods; and 3) consistent MR directions from BMI to metabolite and from metabolite to T2D.



Supplementary Figure S12. Validation of metabolomic signature for T2D prediction across multiple prospective cohorts. In each cohort, we calculated the metabolomic signature scores as depicted in Extended Data Figure 8A. We then examined the AUC improvement comparing a conventional model (Model 1) to a model additionally included the metabolomic signature score (Model 2) on incident T2D prediction, among individuals free of T2D at baseline. The conventional model included age, sex, smoking, lipid-lowering medication use, anti-hypertensive medications, family history of diabetes, hypertension, dyslipidemia, and BMI. AUC from Cox regression was estimated using the median follow-up time.



Supplementary Figure S13. Comparing elastic net regression *vs.* lasso regression on deriving metabolomic signature models in two training sets. The left sub-figure shows the AUC for T2D risk prediction using the metabolomic signature in the Women's Health Initiative (WHI). Cox regression model with elastic net or lasso regularization was fitted to regress incident T2D on metabolites showing FDR<0.05 in metabolome-wide meta-analysis combing all cohorts except WHI. A leave-one-out cross-validation approach was used to acquire unbiased metabolomic signature score for each individual without overfitting. Then a Cox regression was fitted to regress incident T2D on the metabolomic signature score to plot the AUC at the median follow-up time. In the right sub-figure, we presented a sensitivity analysis results, conducted using the Hispanic Community Health Study/Study of Latinos (SOL) as the training set.



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