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Protein-Metabolite Association Studies Identify Novel Proteomic Determinants of Metabolite Levels in Human Plasma

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Conceptualization: REG, MDB, AE; Formal analysis: MDB, AE, UT, DK, SD, AH, XS, SZ, QY; Experiments: MDB, AH, DS; Methodology: MDB, AE, UT, DK, SD, DN, JR, XS, MK, ZY, YG, LF, DS, ZC, DC, MS, AC, RT, PD, KT, YL, WJ, XG, JY, YC, AM, DJ, QY, CB, MS, SR, JR, TW, JW, CC, IS, PN, REG; Software: AE; Writing – original draft: MDB, AE, REG, PN, IS, JW; Writing—review and editing: UT, DK, SD, DN, JR, AH, XS, SZ, MK, ZY, YG, LF, DS, ZC, DC, MS, AC, RT, PD, KT, YL, WJ, XG, JY, YC, AM, DJ, QY, CB, MS, SR, JR, TW, JW, CC, IS, PN, REG, MDB, AE

Declaration of Interests

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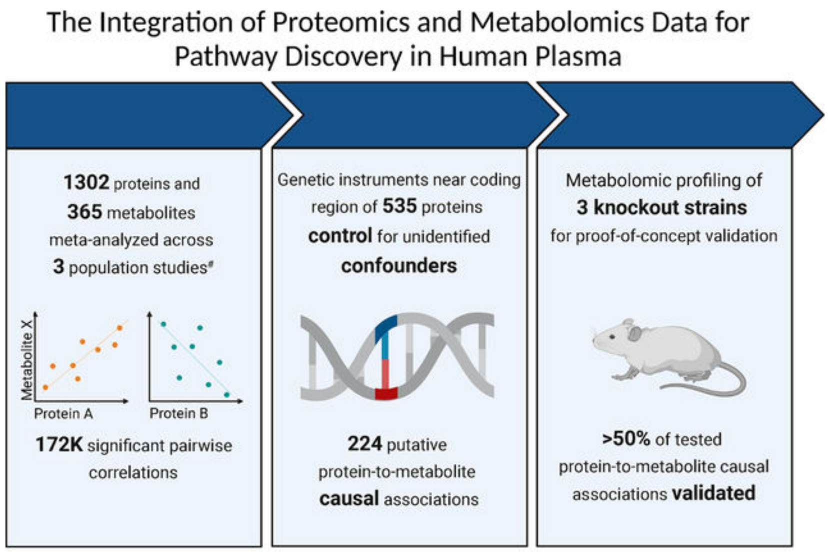
Summary

While many novel gene-metabolite and gene-protein associations have been identified using high throughput biochemical profiling, systematic studies that leverage human genetics to illuminate causal relationships between circulating proteins and metabolites are lacking. Here, we performed protein-metabolite association studies in 3,626 plasma samples from three human cohorts. We detected 171,800 significant protein-metabolite pairwise correlations between 1,265 proteins and 365 metabolites, including established relationships in metabolic and signaling pathways such as the protein thyroxine binding globulin and the metabolite thyroxine – as well as thousands of new findings. In Mendelian Randomization (MR) analyses, we identified putative causal protein-to-metabolite associations. We experimentally validated top MR associations in proof-of-concept plasma metabolomics studies in three murine knockout strains of key protein regulators. These analyses identified previously unrecognized associations between bioactive proteins and metabolites in human plasma. We provide publicly available data to be leveraged for studies in human metabolism and disease.

eTOC Blurb

Benson et al. integrate proteomic, metabolomic, and genomic data in 3,626 individuals from three human cohorts to identify putative causal relationships amongst 1,302 circulating proteins and 365 metabolites in human plasma. Top protein-to-metabolite associations were experimentally validated in plasma metabolomics studies in three murine knockout strains of key protein regulators.

Graphical Abstract



The Integration of Proteomics and Metabolomics Data for Pathway Discovery in Human Plasma

1302 proteins and **365** metabolites meta-analyzed across **3** population studies#

172K significant pairwise correlations

Genetic instruments near coding region of **535** proteins **control** for unidentified **confounders**

224 putative protein-to-metabolite **causal** associations

Metabolomic profiling of **3 knockout strains** for proof-of-concept validation

>50% of tested protein-to-metabolite causal associations **validated**

#Jackson Heart Study, Multi-Ethnic Study of Atherosclerosis, and Health, Risk Factors, Exercise Training and Genetics

Introduction

The integration of metabolomic and proteomic profiling data from large-scale population studies offers the opportunity to connect circulating proteins and metabolites as pathway partners in human physiology. Mass spectrometry-based metabolomics approaches measure low-molecular weight lipids, organic acids, nucleic acids, and other key chemical mediators of central metabolic and signaling pathways. Affinity-based proteomics techniques measure many of the secreted enzymes, transporters, cytokines, peptide hormones, and other proteins that catalyze and regulate these pathways. For example, small molecule profiling techniques can measure thyroxine, while proteomics can measure the transporter thyroxine binding globulin that regulates circulating levels of this metabolite in the thyroid hormone signaling pathway. Similarly, the amino acids aspartate and glutamate, as well as the enzyme aspartate transaminase that catalyzes the interconversion of these two compounds can be assayed by these complementary techniques in the same biological sample. Combining metabolomics and proteomics data may provide insights into new transporter-ligand, enzyme-substrate, and other protein-metabolite pairs that can be used for pathway discovery. However, the systematic integration of metabolomics and proteomics data from large-scale population studies to identify these biological relationships is still in its nascent stages.

Genome-wide association studies (GWAS) of plasma metabolite[1–12] and protein levels[13–28] in large-scale population studies have been increasingly leveraged to identify causal determinants of circulating factors in human plasma. For example, plasma proline levels are strongly associated with genetic variants in the *PRODH* locus that encodes proline dehydrogenase, as well as variants in other enzymes that are important in the catabolism of this metabolite[4,5]. Similarly, GWAS of thrombin protein levels and other blood clotting factors have confirmed pathway relationships within the coagulation cascade[27,29]. While novel gene-metabolite or gene-protein associations have been identified and then validated in experimental model systems[5,15,30–32], systematic studies that leverage human genetics to illuminate novel protein-metabolite associations are lacking.

To begin to determine causal associations between circulating proteins and metabolites, we analyzed mass spectrometry-based metabolomics and aptamer-based proteomics profiling of plasma samples from 3,626 individuals in three cohorts: the Jackson Heart Study (JHS), the Multi-Ethnic Study of Atherosclerosis (MESA), and the Health, Risk Factors, Exercise Training and Genetics (HERITAGE) Family study. In total, we studied the relationships between circulating levels of 1,302 proteins and 365 metabolites measured in the same banked plasma samples. Toward this goal, we first examined correlation data between every pairwise combination of each protein and metabolite and then performed enrichment analyses to detect individual proteins that are significantly associated with specific classes of metabolites. We leveraged the genetic data in each study to perform Mendelian Randomization (MR) analyses to identify putative causal relationships of circulating proteins with metabolite plasma levels. Top protein-to-metabolite MR associations were experimentally validated in proof-of-concept plasma metabolomics studies in three murine knockout models. Further, we provide all protein-metabolite association results as a publicly available dataset for pathway discovery in human metabolism and disease.

Results

We studied the relationships between circulating levels of 1,302 proteins and 365 metabolites measured in fasting plasma samples from participants of the Jackson Heart Study (JHS, n=1,985), the Multi-Ethnic Study of Atherosclerosis (MESA, n=983), and the Health, Risk Factors, Exercise Training and Genetics (HERITAGE) Family study (n=658). Clinical characteristics of the study populations are detailed in Supplementary Table 1a. A list of the studied proteins and metabolites are provided in Supplemental Tables 2 and 3, respectively. An overview of the study design is provided in Figure 1, which included 1) correlation analyses between every pairwise combination of each protein and metabolite, 2) enrichment analyses to detect proteins that are highly associated with specific classes of metabolites, 3) Mendelian Randomization (MR) analyses to identify putative causal relationships of circulating proteins and metabolite plasma levels, and 4) experimental validation of a subset of the top protein-to-metabolite MR associations in proof of concept plasma metabolomics studies in three murine knockout models.

Protein-metabolite correlations in human plasma

Pearson correlation coefficients were calculated for every pairwise protein-metabolite combination within the JHS, MESA, and HERITAGE Family study using age- and sex-adjusted, log-normalized, and standardized protein and metabolite levels. We identified a set of protein and metabolite pairs in each cohort that were significantly correlated with an FDR-adjusted q-value ≤ 0.05 (Figure 2a). Meta-analyses of these correlations across the three studies identified 171,800 significant protein-metabolite correlations (q-value ≤ 0.05) (Supplemental Table 4). Ninety-seven percent of these correlations remained significant when further adjusted for body mass index (BMI), and 87% remained significant when additionally adjusted for estimated glomerular filtration rate (eGFR) to account for potential effects of kidney function on the circulating proteins in multivariable adjusted (MVA) analyses (Figure 2b). Among the 1,614 participants in JHS with available medication histories, 76% of protein-metabolite correlations remained significant when further adjusted for use of antihypertensive (n=992), antidiabetic (n=256), and statin (n=220) medications (Supplemental Figure 1). The magnitude and directionality of correlation coefficients were consistent across the individual studies (Figure 2c).

As anticipated, several of the most significant correlations reflected well-characterized protein-metabolite biological relationships. These included associations between plasma binding proteins such as thyroxine binding globulin and thyroxine (correlation coefficient = 0.51, q-value $\leq 1.0 \times 10^{-300}$), plasma transporters such as apolipoprotein E (APOE) and lipids including diacylglycerol C36:3 (correlation coefficient = 0.51, q-value $\leq 1.0 \times 10^{-300}$), and plasma enzymes such as aspartate transaminase (AST) and its canonical substrate aspartate (correlation coefficient = −0.07, q-value = 8.4×10^{-5}) and product glutamate (correlation coefficient = 0.23, q-value = 5.3×10^{-48}). Visualization of the tens of thousands of additional, previously unexplored protein-metabolite correlations using a heat map demonstrated distinct patterns of correlations between individual proteins and members of specific metabolite classes (Figure 2d). For example, the protein hormone insulin demonstrated positive correlations with metabolites within the carbohydrate, glycerolipid, acyl carnitine, branched chain amino acid, and aromatic amino acid classes and inverse correlations with metabolites within the lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), and polar uncharged amino acid classes (Figure 2e) (Supplemental Table 4). The hormones adiponectin and ghrelin similarly demonstrated strong metabolite correlations within lipid and amino acid class lines. Finally, the hormone fibroblast growth factor 19 (FGF19), a regulator of bile acid synthesis[33], demonstrated marked positive correlations within the bile acid metabolite class. While the clustering of these correlations within specific metabolite classes was consistent with known functions of each of these central metabolic hormones, these analyses also provided novel details regarding interactions with specific subclasses of metabolite species for each protein, particularly in the context of human physiology. For example, insulin demonstrated strong positive correlations with several saturated fatty acids (e.g., butyric acid, q-value = 5.6×10^{-3}) and quinolone carboxylic acids (e.g., xanthurenic acid, q-value = 7.3×10^{-10}), as well as inverse correlations with unsaturated fatty acids (e.g., linoleic acid, q-value = 3.7×10^{-3}), amino fatty acids (e.g., 2-aminoisobutyric acid, q-value = 4.9×10^{-8}), N-acyl amines (e.g., N-oleoyl-glycine, q-value = 8.1×10^{-8}) and dicarboxylic acids (e.g., malonic acid, q-value = 2.9×10^{-3}).

Protein correlations are enriched for specific classes of metabolites in human plasma

To characterize protein-metabolite correlations more systematically, we investigated whether ranked metabolite correlations for each protein were significantly enriched for specific sets of metabolite classes, analogous to Gene Set Enrichment Analyses (GSEA)[34,35]. As an initial proof of concept, we confirmed that the most significantly correlated metabolites with plasma levels of APOE protein were members of the lipid metabolite class, consistent with the well-established role of APOE in lipid transport (Figure 3a). To quantitate this overrepresentation of lipid metabolites among the strongest correlations with APOE, a plot of the running sum statistic for these lipids in the ranked correlation data was used to generate an enrichment score (ES)(Figure 3b). The enrichment score could be assessed for statistical significance from the null hypothesis of no enrichment of correlations for lipid metabolites for APOE using a permutation test as detailed in *Methods*. This analysis was then repeated for each of the 1,302 measured plasma proteins in the dataset.

As shown in Figure 3c, we identified 241 proteins that were significantly enriched for correlations with plasma lipids ($q\text{-value} \leq 0.05$; Supplemental Table 5). These included proteins with well-established roles in lipid metabolism such as proprotein convertase subtilisin/kexin type 9, apolipoprotein B, low-density lipoprotein receptor-related protein 1B, and angiopoietin-like 4. Many novel protein-lipid findings included associations with several members of the cathepsin proteases (CTSA, CTSB, and CTSF), serpin peptidase inhibitors (AGT, SERPING1, and SERPIND1), and secreted glycoproteins (NID1, NID2, LAMA1, and GPC6) (Supplemental Table 5). Further, the broad survey of proteins in this analysis identified additional protein pathway partners that demonstrated enriched correlations for lipid metabolites. A notable example of this included the identification of a pathway node centered on the scavenger receptor CD36, which functions as a high-affinity receptor for long chain fatty acids and other ligands in rodent models and has been implicated in fat metabolism traits in humans[36–40]. The CD36 receptor was not only itself highly enriched for correlations with plasma lipid metabolites ($q\text{-value} = 1.1 \times 10^{-3}$), but two well-established regulatory protein ligands of the receptor, thrombospondin 1[41] and CD5 Molecule Like[42], were also highly enriched for correlations with plasma lipids (THBS1, $q\text{-value} = 8.3 \times 10^{-3}$; CD5L, $q\text{-value} = 4.5 \times 10^{-3}$), highlighting the potential role for this receptor and associated ligands in human lipid metabolism.

We next expanded our analysis to identify proteins with correlations that were enriched for additional major classes of metabolites (Figure 3d–f, Supplemental Table 5). Interestingly, we identified 360 proteins with correlations that were significantly enriched for circulating nucleic acids. Among these were proteins with well-established roles in nucleotide metabolism, such as nucleoside diphosphate kinase B, nucleoside diphosphate kinase A, ectonucleoside triphosphate diphosphohydrolase 1, thymidine kinase 1, and adenylate kinase isoenzyme 1 (Supplemental Table 5). These nucleoside kinases maintain the balance between nucleoside mono-, di-, and triphosphates (e.g., AMP, ADP, and ATP) and several are known to circulate in human plasma with ~1 nM concentrations[43]. While they have been demonstrated to be secreted and to regulate extracellular ATP synthesis in model systems[44–46], their role in human plasma has not previously been fully elucidated.

We also identified 43 proteins with correlations that were significantly enriched for amino and organic acids. Several of these included proteins with well-established roles in the regulation of protein metabolism such as growth hormone receptor, insulin-like growth factor binding protein 2, and adiponectin (Supplemental Table 5). Interestingly, one of the top proteins enriched for correlations with amino and organic acids was the enzyme aminoacylase-1 (ACY1, $q\text{-value} = 2.9 \times 10^{-3}$) which can hydrolyze N-acetyl-amino acids to free amino acids in isolated human and murine plasma[47]. These enrichment data suggest that ACY1 may play a broader role in human plasma amino acid homeostasis, extending prior observations[19,48–51].

Mendelian Randomization analyses identify causal protein-to-metabolite correlations in human plasma.

Pearson correlations do not provide information in regard to the causality or directionality of the relationship between protein and metabolite. To identify potential causal relationships of circulating proteins on metabolite levels in human plasma, we next performed Mendelian Randomization (MR) analyses. This approach leveraged whole genome- or genome-wide association studies (WGAS, GWAS) of each plasma protein and metabolite level in JHS, MESA, and HERITAGE Family study participants. Genetic variants within 1 mega-base (Mb) of the coding gene for each protein ("*cis"* variants) that were independent (linkage disequilibrium $r^2 \leq 0.001$) and strongly associated with circulating levels of the protein (Bonferroni-adjusted p-value ≤ 0.05) were used as instrumental variables to assess whether plasma levels of each protein (exposure) had a causal effect on correlated plasma metabolite levels (outcome) using the Wald method with a single genetic variant and the inverse-variance weighted (IVW) method when multiple genetic variants were available[52–57]. Several methods, including the limited information maximum likelihood (LIML)[58], as well as the median[59], median-weighted[60], and MR-Egger[61] robust methods when instruments contained more than two genetic variants, were used to assess the sensitivity of these analyses, as described in *Methods* and included in Supplemental Table 6.

We found that 547 of the 1302 proteins had *cis* variants that could be used as instrumental variables in MR analyses (Figure 4a) (Supplemental Tables 7–9). Proteins with available *cis* instruments spanned the genome (Figure 4b), and the majority of instruments were located in very close proximity to the transcriptional start site (TSS) of the protein coding gene (Figure 4c). We restricted our analyses to include only instruments in *cis* to the coding gene for each protein so that the effect of these instruments on the metabolite was likely to run through the protein exposure, rather than through an alternative, potentially pleiotropic biochemical pathway[62–64].

In total, we identified 224 putative protein-to-metabolite causal associations between 52 proteins and 146 metabolites that were highly significant (q-value ≤ 0.05) (Figure 4d, Supplemental Table 6). 162 of these associations were identified using the Wald method with a single genetic variant, and 62 of these associations were identified using the IVW method when multiple genetic variants were available. Notably, 58/62 (94%) of the associations that had multiple available genetic variants had concordant weighted median estimates with $p \leq 0.05$, suggesting that a majority of the genetic variants used in these IVW analyses were valid instruments. Similarly, 214/224 (96%) of the associations had concordant LIML estimates with $p \leq 0.05$, suggesting against weak instrument bias. Finally, although potentially under-powered (but consistent with our use of only genetic instruments in *cis* to the coding gene for the protein exposure), 61/62 (98%) of the associations that had multiple available genetic variants had a non-significant MR-Egger intercept test with $p \geq 0.05$, identifying no obvious evidence of horizontal pleiotropy. A complete list of all protein-to-metabolite MR associations that reached nominal levels of significance (p-value ≤ 0.05) is provided in Supplemental Table 6.

Among the top MR findings were several examples of the well-established causal role that APOE protein plays in modulating plasma levels of lipids and fat-soluble vitamins.

These included MR associations between APOE protein and several glycerophospholipids (e.g., C38:5 PE plasmalogen; IVW beta −0.29, q-value 1.9×10^{-7}, LIML q-value 1.0×10^{-6}), phosphosphingolipids, and the lipid-soluble vitamin retinol (Supplemental Table 6).

These analyses also detected strong putative causal associations for several other proteins that were identified to have correlations with lipids, amino and organic acids, and nucleic acids in the enrichment analyses above. For example, we detected strong MR associations between the CD36 scavenger receptor and numerous lipid species not previously associated with this protein, including glycerophospholipids (e.g., C38:7 PE plasmalogen; IVW beta −0.28, q-value 1.2×10^{-15}, LIML q-value 9.6×10^{-15}), acyl carnitines, sphingomyelins, ceramides, and steroids (Figure 4d) (Supplemental Table 6). Notably, we detected strong MR associations between CD36 and several polyunsaturated fatty acids, including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (Supplemental Table 6). These polyunsaturated fatty acids play a key role in eicosanoid signaling. CD36 has been implicated in the cellular uptake of polyunsaturated fatty acids using *in vitro* models[65,66]. However, these findings may suggest a broader role for CD36 as a central regulator of lipid homeostasis in human plasma.

There were also strong MR associations between the enzyme ACY1 and several N-acetyl amino acids, including N-acetyl-glutamate (IVW beta −1.25, q-value 1.9×10^{-33}, LIML q-value 4.5×10^{-12}), N-acetyl-alanine, N-acetyl-glutamine, and N-acetyl-serine (Figure 4d) (Supplemental Table 6). Circulating levels of N-acetyl amino acids are known to be tightly regulated and have recently been tied to several cardiometabolic phenotypes in human population studies, such as insulin resistance[47], incident coronary artery disease[67], and incident heart failure[68]. These MR results are consistent with the known role of ACY1 in modulating the levels of these N-acetyl-amino acids in human plasma.

Intriguingly, proprotein convertase subtilisin/kexin type 9 (PCSK9) demonstrated putative causal associations with multiple acyl carnitines, including CAR 18:1 (IVW beta −0.52, q-value 5.8×10^{-4}, LIML q-value 1.5×10^{-3}), CAR 18:2, CAR 16:0, and CAR 14:1 (Figure 4d) (Supplemental Table 6). Acyl carnitines were among the most strongly associated metabolites with PCSK9 protein in pairwise Pearson correlation analyses (e.g., CAR 18:1 correlation coefficient −0.26, q-value 6.5×10^{-64}) (Supplemental Table 4). Carnitines play a key role in the regulation of energy metabolism by facilitating the transport of long-chain fatty acids from adipose tissues to target cells. While PCSK9 has recently been shown to reduce the uptake of long-chain fatty acids by adipocytes in a cell culture system[69], these MR association data suggest a potential role for PCSK9 in regulating the carnitine transport system in human plasma.

Protein-to-metabolite causal associations predicted by MR analyses in human plasma experimentally validated in three murine knockout models.

As a proof of concept to test the causal protein-to-metabolite associations predicted by MR analyses above, we conducted plasma metabolomics on available C57BL/6 murine knockout (KO) strains for the three proteins that had the most significant MR metabolite associations (CD36, APOE, and ACY1) and compared these to wild-type (WT) controls.

The CD36 scavenger receptor was predicted to have a causal association with 68 metabolites in the human MR analyses above (q-value ≤ 0.10) (Supplemental Table 6), 50 of which were also measured in the *CD36* KO mouse. We identified significant differences in the plasma levels of 27 of these metabolites (54%) in metabolomic profiling studies of the *CD36* KO animals (n=6) versus WT controls (n=8; p ≤ 0.05) (Figure 5a). These included experimental validation of predicted causal relationships with specific glycerophospholipids, sphingolipids, and fatty acyls, including causal associations of CD36 with circulating levels of the central signaling fatty acids docosahexaenoic acid (DHA; *CD36* KO/WT fold-change = 0.73 ± 0.06, p-value = 0.01) and arachidonic acid (*CD36* KO/WT fold-change = 0.74 ± 0.04, p-value = 0.01) (Figure 5b)(Supplemental Table 10).

Similarly, APOE was predicted to have causal associations with 13 metabolites in the human MR analyses (q-value ≤ 0.10) (Supplemental Table 6), and we detected significant differences with the expected directionality in the plasma levels of four of these metabolites in *APOE* KO animals (n=6) versus WT controls (n=8; p≤0.05) (Figure 5c). Notably, each of the four experimentally validated metabolites were phosphatidylethanolamine (PE) plasmalogens, including C38:6 (*APOE* KO/WT fold-change = 1.30 ± 0.05, p-value = 9.47×10^{-4}), C38:5, C36:3, and C40:7 PE plasmalogens (Figure 5d)(Supplemental Table 10). PEs have been speculated to interact with APOE following the hepatic secretion of nascent very low density lipoprotein (VLDL) particles in cell-based *in vitro* studies[70,71]. These findings may suggest a causal role for APOE in the regulation of circulation PE plasmalogen levels in human plasma.

Finally, the circulating enzyme ACY1 was predicted to have causal associations with five metabolites in human MR studies, and we detected directionally-consistent significant differences in the plasma levels of four of these metabolites (80%) in *ACY1* KO animals (n=6) versus WT controls (n=6; p ≤ 0.05)(Figure 5e), including N-acetyl-glutamate (*ACY1* KO/WT fold-change = 7.19 ± 0.57, p-value = 1.11×10^{-6}), N-acetyl-glutamine, N-acetyl-serine, and N-acetyl-alanine (Figure 5f)(Supplemental Table 10).

In total, we experimentally validated 35 of the 68 (51%) predicted protein-to-metabolite MR associations. Further, we experimentally validated 62 additional protein-metabolite associations with significant pairwise Pearson correlations (q ≤ 0.05) that either did not have an available MR instrumental variable or were not captured by MR analyses (Supplemental Table 10). These data may provide new insight into potential downstream biological pathways that connect disease-associated proteins to end clinical phenotypes that can be further investigated at the bench. Association analyses between ACY1, APOE, and CD36 and cardiometabolic traits are provided in Supplemental Table 11.

Discussion

This study leveraged metabolomic and proteomic profiling of plasma samples from three human cohorts to determine if the integration of these datasets may identify novel causal relationships between specific circulating proteins and metabolites. The profiling data from the JHS, MESA, and HERITAGE Family studies were "harmonized," in that we used the same mass spectrometry-based metabolomics and aptamer-based proteomics platforms in parallel across each of the 3,626 samples, providing an ideal opportunity to perform protein-metabolite association studies. Additionally, genomic data were available for each participant, allowing for the study of putative protein-to-metabolite causal associations with Mendelian Randomization analyses and follow-up studies in a select group of knockout mice. This analysis provides several important initial insights into the integration of metabolomic and proteomic profiling data for pathway discovery.

First, we show that known protein-metabolite associations that are key to established metabolic and signaling pathways (e.g., thyroxine binding globulin protein and thyroxine metabolite) can be detected in banked samples from population studies. Further, as might be expected for proteins and metabolites that are related though a shared biological pathway, these associations persist despite adjustment for broad baseline characteristics of study participants (e.g., age, sex, BMI, eGFR, medication use) and are reproducible across studies conducted at different geographical locations at different times, and in participants of diverse race and ethnicity. Finally, the directionality of these associations may provide insight into the biological relationship between each protein and metabolite. For example, we detected strong correlations between the enzyme aspartate transaminase in the biologically-expected negative direction with its catalytic substrate aspartate and positive direction with its catalytic product glutamate (higher protein enzyme levels are associated with lower metabolite substrate and higher metabolite product levels. It is important to note that these correlation data reflect a single cross-sectional point in time, however, and the directionality of certain protein-metabolite relationships may change over different physiological (and pathophysiological) states.

Second, this study leverages protein-metabolite association data to link protein-metabolite relationships previously identified in model systems to human biology. We used protein-metabolite correlation data to perform enrichment analyses and identify several examples of protein-lipid, protein-amino acid, and protein-nucleic acid associations that have been studied in cell- and animal-based systems, but that have not to our knowledge been previously demonstrated in human plasma. For example, the secreted protease Cathepsin B has emerged as a potential novel lipid regulatory protein in several experimental model systems. Knockout of the Cathepsin B gene results in marked improvements in liver triglyceride and blood total cholesterol levels in a murine model of nonalcoholic fatty liver disease[72]. Mechanistically, Cathepsin B has been shown in cultured cell-based model systems to regulate very-low-density lipoprotein (VLDL) secretion and free fatty acid uptake by cleaving liver fatty acid-binding protein (LFAB)[73]. The protein-metabolite enrichment analyses presented here extend these experimental findings and provide strong rationale for further study of Cathepsin B in human plasma lipid homeostasis.

Similarly, extensive experimental data have demonstrated a key role for adenylate kinase and ecto-nucleoside diphosphokinase nucleotide conversion enzymes in the regulation of extracellular ATP levels in cultured hepatocytes[74], endothelial cells[75], and lymphoid cells[75], as well as in human vitreous fluid[76]. The protein-metabolite association data in the current study build on these mechanistic observations and suggest a role for these enzymes in the regulation of extracellular ATP and purine signaling that is reflected in human plasma. Importantly, while we used an enrichment analysis strategy based on GSEA[34,35] to interrogate the protein-metabolite association data in this study, additional methods could be employed to query these extensive data for pathway discovery. Thus, we have made the complete protein-metabolite association study dataset publicly available for further analyses.

Third, this study demonstrates how Mendelian Randomization analyses can be leveraged to "triage" putative causal protein-to-metabolite associations from protein-metabolite correlation data for further experimental study. By integrating genetic data with our protein-metabolite association findings, we were able to use genetic variants located in or near the coding gene for a measured protein to examine the causal effect of that protein exposure on a metabolite outcome in human plasma. Approximately 40% of the measured proteins in our studies had strong, independent associations with genetic variants in *cis* to the protein cognate gene that could be used as instruments in MR analyses. Notably, the use of three cohorts representing diverse race/ethnicities and minor allele frequencies improved the ability to identify MR instruments. For example, the inclusion of African Americans in the JHS highlighted the variant rs2229152 (JHS MAF=1.7%) as an instrument for circulating ACY1 protein. This missense variant has been linked to the rare autosomal recessive inborn error of metabolism ACY1 deficiency that often manifests with neurologic symptoms in humans (MIM 609924)[77], was strongly associated with circulating levels of ACY1 protein in JHS, and provided an MR instrument to support putative causal roles for ACY1 protein on plasma levels of N-acetyl-glutamate, N-acetyl-glutamine, N-acetyl-serine, and N-acetyl-alanine in JHS, each of which was experimentally validated in our murine *ACY1* knockout studies. This variant is too rare in European populations (ALFA European MAF=0.0003) to have been captured for study in the MESA (MAF=0.006) or HERITAGE Family study (MAF=0.004), and thus would not have been available for analysis if not for inclusion of the JHS. This suggests that as an increasing number of multi-omics studies in diverse populations become available, our ability to study biologically significant protein-metabolite relationships will also improve.

It is also notable that over half (51%, 35 of 68) of the tested protein-to-metabolite MR associations experimentally validated in our three murine knockout models with at least nominal levels of significance ($p \leq 0.05$). This suggests that a significant fraction of the 224 total protein-to-metabolite MR associations that we have identified point to biological relationships that can be further elucidated in model systems. Further, 62 additional protein-metabolite associations that were identified in the pair-wise Pearson correlation analyses but either did not have an available MR instrumental variable or were not captured by MR analyses validated in the murine models. This indicates that the protein-metabolite association data may highlight a substantial number of additional, biologically significant relationships, and that MR and Pearson correlation data can identify both overlapping and distinct causal associations. In terms of the protein-to-metabolite MR associations that did not experimentally validate, there were examples of strong MR associations that closely missed statistically-significant experimental validation in the studied knockout models. For example, we identified an MR association between the fatty acid scavenger receptor CD36 and the fatty acid EPA in humans with an IVW beta of 0.17 and a q-value of 8.8×10^{-5} that closely missed the statistical threshold for validation in the CD36 knockout studies (CD36 KO/WT fold-change = 1.31 ± 0.06, p-value = 0.08). It is possible that this relationship would have experimentally validated with increased statistical power. Whether our findings reflect the specific experimental conditions of our studies (e.g. 5-week old male mice maintained on a normal chow diet) or bona-fide biological differences in protein-metabolite relationships between the human populations and mouse models that we analyzed will be the subject of future study.

We have identified several specific examples of protein-metabolite associations that suggest novel regulatory mechanisms affecting plasma metabolites. These included experimental validation of 27 (54%) of the predicted MR associations between the scavenger receptor CD36 protein and levels of plasma lipid metabolites. CD36 has been well-documented to regulate fatty acid uptake in a number of cell types and rodent models, including hematopoietic stem cells[78], leukemic stem cells[79], cardiac myocytes[80], adipocytes[81], endothelial cells[81], and macrophages[82]. CD36 is further known to regulate plasma levels of non-esterified fatty acids in murine models[83]. Our experimentally-validated protein-metabolite association data extend these findings and suggest that CD36 may play a central role in regulating plasma levels of a range of glycerophospholipid, sphingolipid, and fatty acyl metabolites. These include the polyunsaturated fatty acids arachidonic acid and docosahexaenoic acid (DHA), two key metabolites that participate in a wide array of eicosanoid-mediated biological pathways important in inflammation, nociception, the immune response, cell growth, atherosclerosis, and blood pressure regulation. DHA has furthermore been used clinically to reduce the risk of coronary heart disease, hypertension, and hypertriglyceridemia. While CD36 has been implicated in the regulation of polyunsaturated fatty acid cellular uptake using cultured cell-based *in vitro* models[65,66], these findings provide a rationale for further studies of the role of CD36 in regulating key lipid metabolites in human plasma.

Finally, we note that circulating levels of ACY1, APOE, and CD36 proteins are strongly associated with several cardiometabolic traits in humans. For example, plasma levels of ACY1 and APOE have previously been associated with the future development of type 2 diabetes (T2D) in healthy, non-diabetic individuals[47,84]. Interestingly, several of the metabolites that are predicted to be downstream of ACY1 and APOE in the current MR analyses have also been associated with the future risk of T2D in the same populations. For example, plasma levels of N-acetyl-alanine are associated with future T2D in participants of the JHS[85] and are modulated by ACY1 in our human MR analyses and in murine *ACY1* knockout studies. Similarly, plasma levels of C36:3 PE plasmalogen and C34:3 PC plasmalogen are associated with future T2D in JHS[85] and modulated by APOE in our human MR analyses and murine *APOE* knockout studies. These MR data may provide new insight into potential downstream biological pathways that connect disease-associated proteins to end clinical phenotypes that can be further investigated at the bench.

In summary, we demonstrate that the integration of proteomic and metabolomic profiling data can be used to identify novel protein determinants of circulating metabolite levels in human plasma. We provide proof-of-concept that these insights can be tested successfully in model systems. Finally, we are making all protein-metabolite association data from three large human cohorts available as a public resource for the further study of human metabolism.

Limitations of Study

Our study had several limitations. Although the metabolomics and proteomics platforms applied in these studies provide broad coverage, they are targeted platforms that include sentinel proteins and metabolites designed to survey a wide array of biological processes and do not provide a complete catalogue of every circulating protein and metabolite species in human plasma. The proteomics platform is further agnostic to post-translational changes in proteins. We expect that our ability to refine these initial association data for pathway discovery will improve as platform coverage improves. Similarly, although our study used "harmonized" metabolomics and proteomics data across three human cohort studies, the sample size is relatively modest compared with many GWAS. We expect that insight from protein-metabolite association studies will improve as increasing numbers of multi-omics studies become available, especially in populations including diverse ethnicities and races. It should be noted that we were only able to perform MR analyses on the 42% of proteins with available *cis* instrumental variables. We limited our analyses to include only *cis* instruments to limit the risk for horizontal pleiotropy and to validate the specificity of the affinity-based aptamer, but note that many additional causal protein-to-metabolite relationships likely exist within our data. Finally, while the use of *cis* instruments provided a biologically-plausible link to the protein exposure and thus allowed for the identification of putative causal association in the direction of protein-to-metabolite, we were not able to definitively assess for possible causal associations flowing in the opposite direction from metabolite-to-protein. In exploratory studies, we attempted to perform bidirectional (i.e. metabolite-to-protein) MR analyses for the 146 metabolites that we identified in our protein-to-metabolite MR studies above. A challenge in conducting metabolite-to-protein MR analyses has been in identifying genetic instruments that have as clear a biological tie to the metabolite exposure as is the case for a protein exposure (which has a coding gene). Despite a systematic search using data from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Maps, we were unable to identify candidate genetic instruments for these metabolites that were located in *cis* (1Mb upstream or downstream) to genes that encoded enzymes with a biological link to the metabolite exposure (e.g., the gene *MAOA*, which encodes the enzyme serotonin deaminase, for the metabolite exposure serotonin). We anticipate that our ability to perform metabolite-to-protein MR associations will improve as larger multiomics datasets becomes available.

STAR Methods

Resource Availability

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Robert E. Gerszten, MD (rgerszte@bidmc.harvard.edu).

Materials Availability—This study did not generate new unique reagents.

Data and Code Availability

- Individual-level metabolomic, proteomic, and genomic data from JHS, MESA, and the HERITAGE Family study are available through application to the respective cohorts. All protein-metabolite association data, including pairwise Pearson correlation, enrichment, and Mendelian Randomization data, are included in the article and Supplemental Data. An excel file containing the values that were used to create all graphs in the article are available in Data S1 – Source Data. Data are also available through a Shiny app user interface that can be accessed through the following link: https://github.com/aeisman/protein-metabolite. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

- All analyses except where specifically noted were performed using code written in The Julia Programming Language[86] and R project for statistical computing. All original code has been deposited at https://github.com/aeisman/protein-metabolite and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Study Participant Details

Human Cohort Study Participants—The JHS, MESA, and the HERITAGE Family studies have been previously described[87–89]. Briefly, JHS is a community-based longitudinal cohort study that started in 2000 and included 5306 self-identified Black individuals from the Jackson, Mississippi metropolitan area. Proteomic profiling was performed on fasting baseline plasma samples from 2143 individuals; 399 samples were from a nested case-cohort study of incident coronary artery disease and the remaining were randomly selected from individuals with available plasma samples, as previously described[90]. Metabolomic profiling was performed on 2750 individuals as nested case-control studies for coronary disease (n=400) and chronic kidney disease (759) with the remaining samples randomly selected (n=1,591), as previously described[68]. Samples from 1985 individuals from JHS had available baseline metabolomics, proteomics, and genomics data from Visit 1 and were included in the present study. Baseline characteristics of the participants with available multi-omics profiling were comparable to the broader JHS population, as shown Supplemental Table 1b. MESA is a population-based study that started in 2000 and included 6814 self-identified White, Black, Hispanic, and Asian individuals recruited from six clinical centers across the United States. Samples from 983 randomly-selected individuals with available baseline metabolomics, proteomics, and genomics data from Visit 1 were included in the present study. The HERITAGE Family study is an exercise training study that started in 1994 and included 763 self-identified White and Black individuals in family units recruited from four clinical centers across the United States and Canada. Samples from 658 individuals with available baseline metabolomics and proteomics data from Visit 1 were included in the present study.

Study Approval—The JHS human study protocol was approved by the Jackson State University, Tougaloo College, and the University of Mississippi Medical Center Institutional Review Boards, and all participants provided written informed consent. The MESA human protocol was approved by The Lundquist Institute (formerly Los Angeles BioMedical

Research Institute) at Harbor-University of California, Los Angeles Medical Center, University of Washington, Wake Forest School of Medicine, Northwestern University, University of Minnesota, Columbia University, Johns Hopkins University, and University of California, Los Angeles Institutional Review Boards, and all participants provided written informed consent. The HERITAGE Family study human study protocol was approved by the Institutional Review Boards at the Beth Israel Deaconess Medical Center, University of Washington, and the four clinical centers of the HERITAGE Family study, and all participants provided written informed consent. All animal experiments were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Animal Studies—Plasma was collected for LC-MS studies by cardiac puncture from the following fasting 5-week old, male mice maintained on a normal chow diet in housing conditions with a 14-hour light/10-hour dark cycle and temperatures of 18–23 deg C with 40–60% humidity: B6.129P2-Apoetm1Unc/J (RRID:IMSR_JAX:002052, obtained from Jackson Labs), B6.129S1-Cd36tm1Mfe/J (RRID:IMSR_JAX:019006, obtained from Jackson Labs), C57BL/6N-Acy1em1(IMPC)J/Mmucd (RRID:MMRRC_046467-UCD, obtained from The Knockout Mouse Project (KOMP)), and C57BL/6J (RRID:IMSR_JAX:000664, obtained from Jackson Labs). Homozygous APOE and CD36 knockout animals were compared to wild-type C57BL/6J controls. Homozygous ACY1 knockout animals were compared to wild-type littermates. LC-MS was conducted using the same methods as described above.

Method Details

Proteomic Profiling—Aptamer-based proteomic profiling methods using the SOMAscan platform have been described previously[91–93]. Briefly, in each study, proteomics was performed on baseline plasma samples that were collected during Visit 1 in EDTA tubes and subsequently stored at −70 degrees C. The SOMAscan 1.3k platform was used in JHS and MESA studies, and the SOMAscan 5k platform was used in the HERITAGE Family study. Only proteins included in the 1.3k platform were used for this analysis in HERITAGE. A list of SOMAmer IDs and corresponding protein targets is included in Supplemental Table 2.

Metabolomics Profiling—Metabolomics profiling was performed using liquid chromatography mass spectrometry (LC-MS) on fasting baseline plasma samples that were collected during Visit 1 in JHS, MESA, and the HERITAGE Family study, as previously described[68,94]. Briefly, amino acids, amines, acylcarnitines, lipids, and other water-soluble, polar metabolites were measured using a Nexera X2 U-HPLC (Shimadzu) equipped with a 150 × 2 mm, 3 μm Atlantis hydrophilic interaction LC column (Waters) coupled to a Q Exactive hybrid quadrupole Orbitrap MS (ThermoFisher Scientific). Metabolites were extracted from 10 μl plasma by adding 90 μl of Acetonitrile:Methanol:Formic acid (74.9:24.9:0.2,v/v/v) solution spiked with valine-d8 (Sigma) and Phenylalanine-d8 (Cambridge Isotope Laboratories). The metabolites were eluted at 0.25 ml/min with 5% buffer A (10 mM Ammonium-Formate, 0.1% formic acid in water) for 0.5 minutes followed by a linear gradient to 40% buffer B (0.1% formic acid in acetonitrile) over 10 minutes. MS analyses were carried out using electrospray ionization in the positive mode and full scan spectra were acquired over 70–800 m/z. Raw data were processed using Trace Finder (v3.3, Thermo Fisher Scientific) and Progenesis QI (Waters). Sugars, purines, pyrimidines, organic acids and other intermediary metabolites were measured using a 1290 Infinity LC system (Agilent Technologies) equipped with a 100 × 2.1 mm XBridge amide column (Waters) coupled to a 6490 Triple Quad MS (Agilent Technologies) in negative ionization mode via multiple reaction monitoring (MRM) scanning. Data were quantified using MassHunter Quantitative Analysis software (V10.1, Agilent).

To ensure quality control, a mixture of ~150 reference standards was analyzed before, during periodic intervals throughout, and after each MS run to ensure reproducibility of LC retention times, LC peak shapes, and MS sensitivity. Isotope labeled internal standards were monitored in each sample throughout the duration of each run. Pooled plasma samples were monitored after every 10 participant samples to standardize for MS drift over time using "nearest neighbor" normalization and between batches. Separate pooled plasma samples were monitored after every 20 participant samples to determine coefficient of variation (CV) for each metabolite. Metabolite identities were confirmed using authentic reference standards. All metabolite peaks were manually reviewed for peak quality in a blinded manner. None of the included metabolites had poor peak quality or CVs ≥ 30% averaged across batches. A complete list of metabolites included in this study is included in Supplemental Table 3.

Genotyping—Whole-genome sequencing (WGS) in JHS and MESA has been described[95]. Participant samples underwent >30× WGS through the Trans-Omics for Precision Medicine project at the Northwest Genome Center at University of Washington and joint genotype calling with participants in Freeze 6. Genotype calling was performed by the Informatics Resource Center at the University of Michigan. Genotyping in HERITAGE was performed on the Illumina Infinium Global Screening Array, and genotypes were called using Illumina's GenCall based on the TOP/BOT strand method. Genotype imputation to the TOPMed Freeze5 reference panel was performed using the University of Michigan Imputation Server Minimac4. Phasing was performed with Eagle v2.4. Sites with call rate <90%, mismatched alleles, or invalid alleles were excluded.

Genome-Wide Association Studies—Metabolite and protein levels in the JHS, MESA, and HERITAGE Family Study were log-transformed, scaled to a mean of zero and standard deviation of 1, and residualized on age, sex, batch (for metabolites), plate (for proteins), and principal components of ancestry 1–10 as determined by the Genetic Estimation and Inference in Structured samples (GENESIS)[95]. These values were inverse normalized and tested for association with genetic variants using linear mixed effects models adjusted for age, sex, the genetic relationship matrix, and principal components of ancestry 1–10 using the fastGWA model implemented in the GCTA software package.

Correlation Analyses—Metabolite and protein levels were log-transformed, scaled to a mean of zero and standard deviation of 1, and residualized on age, sex, batch (for metabolites), and plate (for proteins). Additional models included further adjustment for body mass index (BMI) and estimated glomerular filtration rate (eGFR; not available in HERITAGE Family study), where indicated. Pearson correlation coefficients were calculated for each pairwise protein-metabolite combination within each study and meta-analyzed across studies using the metacor function within the General Package for Meta-Analysis in R[96]. A correlation heat map was generated using the Heatmap3 R package[97], in which the organization of metabolites was fixed by RefMet superclass, main class, and subclass[98], and proteins were allowed to self-organize using the default complete linkage method of the hierarchical clustering function.

Enrichment Analyses—Enrichment analyses were performed to identify proteins with pairwise metabolite correlations that were enriched for a specific metabolite class using a method analogous to Gene Set Enrichment Analysis (GSEA) [34,35]. Sets of metabolites were generated according to RefMet superclasses, with fatty acyls, glycerolipids, glycerophospholipids, prenol lipids, sphingolipids, and sterol lipids combined into a single, combined lipid set. Members of the lipid set were evaluated in the lipid enrichment analysis and not included in other metabolite set enrichment analyses due to the large size of this set.

Meta-analyzed metabolite correlation results for each protein were ranked by p-value, annotated by metabolite RefMet class set, and a running sum statistic was calculated to generate an enrichment score (ES). The running sum statistic increased when it encountered a member of the analyzed RefMet metabolite class set and decreased when it encountered a nonmember of the analyzed set. Each increase was weighted by the strength of the metabolite correlation with the protein (according to p-value) normalized by the sum of the correlations over all the metabolites (p=1 in equation 1)[35]. The significance of each ES was assessed by comparison to the null distribution of calculated ES for each metabolite class generated by running 100000 simulations of the analysis.

Mendelian Randomization Analyses—Genetic instrumental variables (IV) for MR were selected from variants that were located in *cis* to the coding gene for each protein (≤ 1 million bases upstream or downstream of the transcriptional start site for the protein cognate gene, or to the transcription end site for genes > 1 million bases) that had a study-specific observed minor allele frequency (MAF) ≥ 0.01 and were associated with circulating levels of the measured protein with a p ≤ 0.05 that was Bonferroni-adjusted for the total number of variants within this *cis* window. Candidate instruments that met these criteria were then pruned using a study-specific linkage disequilibrium (LD) threshold of 0.001 with PLINK 1.9[99–101]. A complete list of MR IVs used in this study is available in Supplemental Tables 7–9. One-sample MR using individual level data was performed since genetic variants, plasma protein levels, and plasma metabolite levels were available in the same individuals, and because samples between studies represented different ethnic groups with different patterns of linkage disequilibrium, minor allele frequencies, and population characteristics (Supplementary Table 1). Pairwise significant associations from meta-analyzed Pearson correlation and enrichment analyses were considered candidate protein-to-metabolite causal associations for MR. Causal effect estimates were obtained using the inverse-variance weighted (IVW) method using the MendelianRandomization R package[102] and then meta-analyzed across all three studies using a fixed effect model[96]. The limited information maximum likelihood (LIML) robust method was performed to assess the sensitivity for findings, and further supplemented with MR-Egger, median, and median-weighted methods when instruments contained more than two genetic variants (Supplemental Table 6). MR was performed on each protein that had ≥ 1 available IV and associated metabolites with q-value ≤ 0.05 (either by pairwise correlation analysis or enrichment analysis).

Quantification and Statistical Analyses

Reported p-values were estimated using the Fisher transformation. Throughout the manuscript, significance levels were adjusted for multiple hypothesis testing by computing Benjamini-Hochberg FDR-adjusted q-values for each protein using the Bioconductor q-value package in R[103]. The significance of metabolite associations was calculated for each protein so that findings would remain agnostic to the specific proteomic platform used in each study, and to establish an analytical pipeline that will be scalable to the addition of future datasets that may use different proteomics platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Jackson Heart Study

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HERITAGE

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Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research.

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Highlights

- Integrating human plasma proteomic and metabolomic data informs pathway discovery
- Genomic data can help identify putative protein-to-metabolite causal associations
- Top protein-metabolite causal associations validated in experimental mouse models
- Protein-metabolite association data have been made publicly available

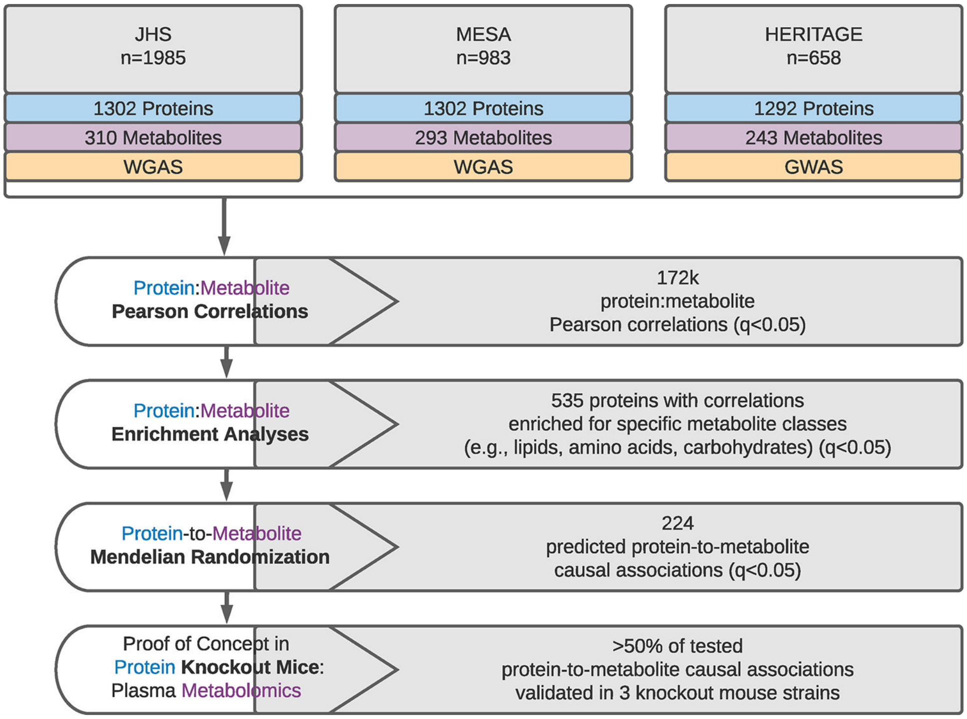

Figure 1. The integration of human plasma proteomic, metabolomic, and genomic profiling data for pathway discovery.

Flow diagram detailing the experimental pipeline and main results from the integration of plasma proteomic, metabolomic, and genomic profiling datasets in the Jackson Heart Study (JHS), Multi-Ethnic Study of Atherosclerosis (MESA), and Health, Risk Factors, Exercise Training and Genetics Study (HERITAGE Family study). WGAS = whole genome association study, GWAS = genome wide association study.

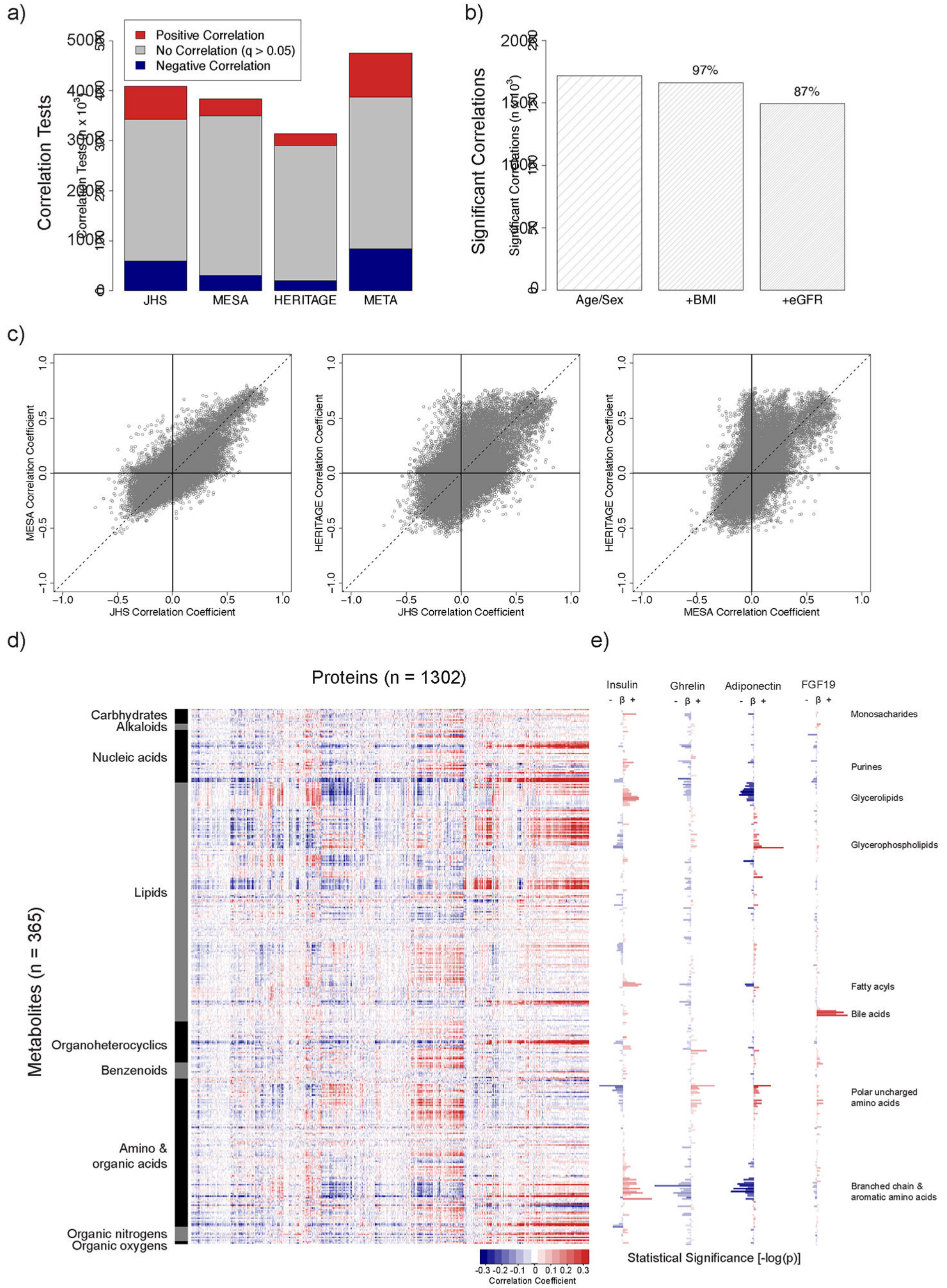

Figure 2. Protein-metabolite correlations in human plasma.

Pearson correlation coefficients were calculated for every pairwise protein-metabolite combination within the JHS, MESA, and HERITAGE Family study using age- and sex-adjusted, log-normalized, and standardized protein and metabolite levels. (A) A subset of proteins and metabolites were positively (red) or negatively (blue) correlated with an FDR-adjusted q-value ≤ 0.05 in each study, and in a meta-analysis of the three studies. (B) Ninety-seven percent of the meta-analyzed age- and sex-adjusted protein-metabolite correlations remained significant with an FDR-adjusted q-value ≤0.05 when further adjusted for BMI, and 87% remained significant when additionally adjusted for eGFR. (C) The magnitude and directionality of the meta-analyzed age- and sex-adjusted protein-metabolite correlations were consistent across studies. (D) Visualization of the protein-metabolite correlations using a heat map demonstrated distinct patterns of associations between individual proteins and members of specific classes of metabolites. Individual metabolites were ordered according to RefMet class along the y-axis, and proteins were allowed to order using a hierarchical cluster analysis along the x-axis. The magnitude and directionality of the correlation coefficient for each protein-metabolite association is depicted by color, as indicated in the legend. (E) The statistical significance (−log(p-value)) of the correlation between each metabolite and four representative protein hormones is shown.

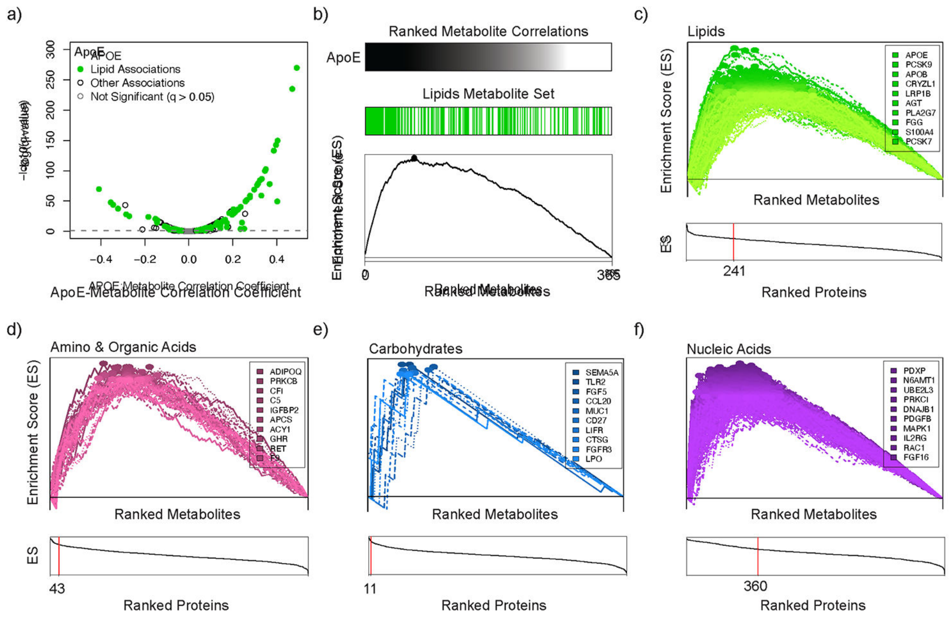

Figure 3. Protein correlations are significantly enriched for specific metabolite classes.

(A) A volcano plot demonstrates that the most significantly correlated metabolites with plasma levels of APOE protein were members of the lipid metabolite class. (B) To evaluate this enrichment for lipids more quantitatively, an enrichment score (ES) was computed and visualized as the maximum point (marked with ●) of a running sum statistic that increases proportionally with each lipid and decreases with non-lipids along the ranked list of metabolite correlations with plasma levels of APOE. (C) 241 proteins were significantly enriched for correlations with plasma lipids (FDR-adjusted q-value ≤ 0.05). In the top panel, the ES tracings are shown for each individual protein, and the ten proteins with the highest enrichment scores are listed. In the bottom panel, proteins are ordered in descending order of calculated enrichment scores (x-axis), and the number of proteins significantly enriched for correlations with lipids (FDR-adjusted q-value ≤ 0.05) is indicated with the vertical red line. Similar enrichment analyses are shown for Amino and Organic Acids (D), Carbohydrates (E), and Nucleic Acids (F). The complete enrichment analysis dataset is available in Supplemental Table 5.

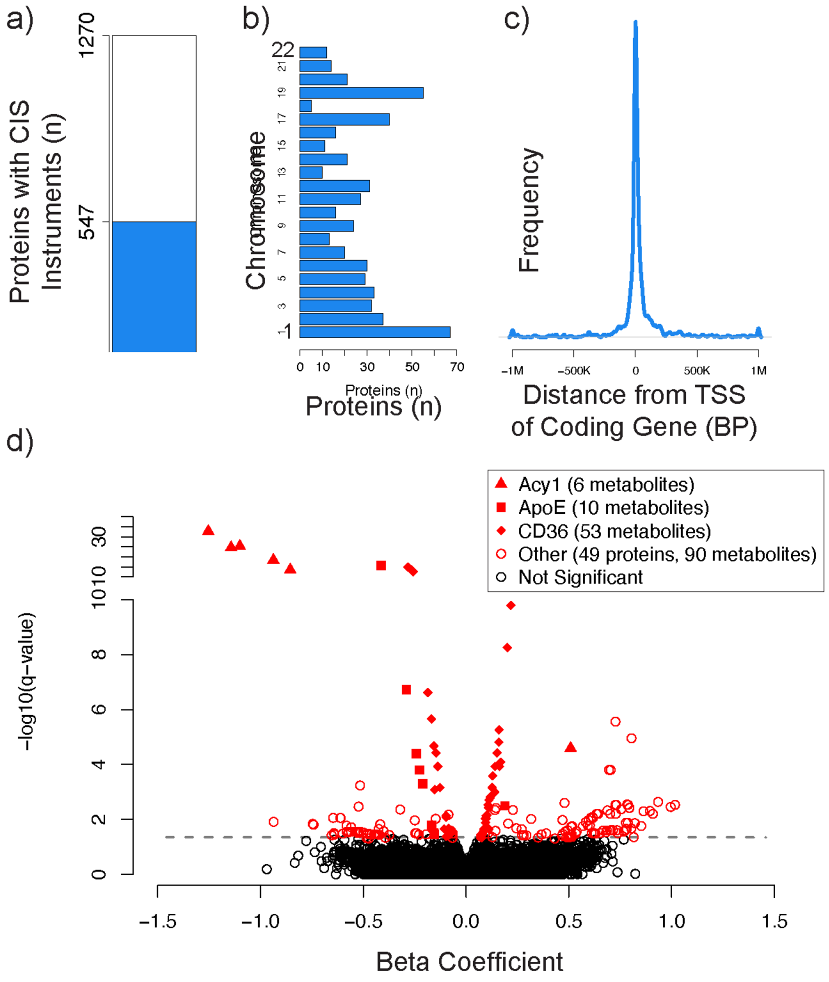

Figure 4. Mendelian Randomization analyses identify putative causal protein-to-metabolite associations in humans.

Proteins with at least one pQTL in *cis* (located within 1Mb) of the protein coding gene that could be used as an instrumental variable (IV) in Mendelian Randomization (MR) analyses are depicted in blue (a). The proteins with available *cis* instruments were distributed evenly across the genome (b). pQTLs used in IVs were generally located near the transcriptional start site (TSS) of the protein coding gene (c). A volcano plot depicts the 224 significant MR associations between 52 proteins and 146 metabolites with an FDR-adjusted q-value ≤ 0.05 (d). The three proteins with the most significant MR metabolite associations are depicted by distinct shapes described in the figure legend.

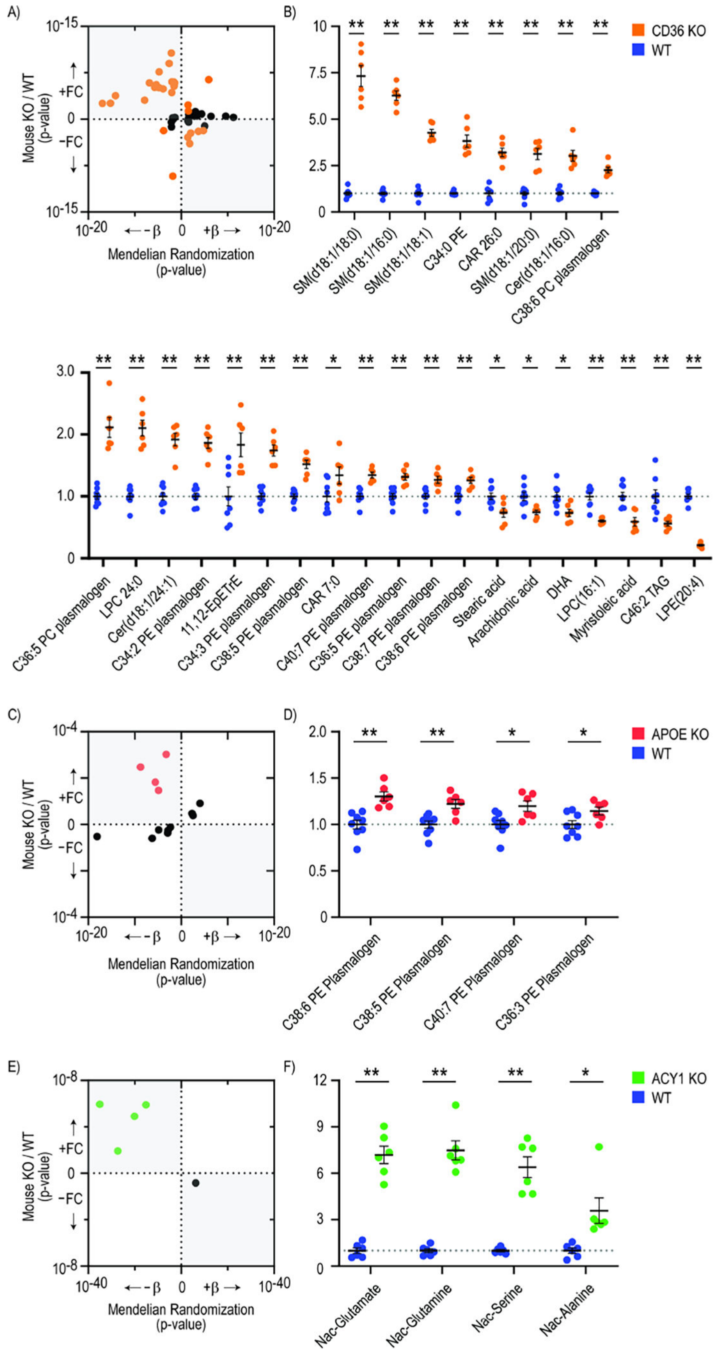

Figure 5. Protein-to-metabolite causal associations predicted by Mendelian Randomization analyses in humans experimentally validate in murine knockout models.

Plasma metabolomics was conducted on C57BL/6 murine knockout (KO) strains for CD36 (n=6), APOE (n=6), and ACY1 (n=6) and compared to wild type (WT) controls (n=8). Scatterplots depict the number of predicted protein-to-metabolite MR associations in humans (with q≤0.1) that validated in each murine model (with p≤0.05, highlighted in color), as well as the concordance in directionality of these associations (a, c, e). The position on the x axis represents the p-value of the predicted MR association between each protein and metabolite level in the human studies. The position on the y axis represents the p-value of the difference in metabolite level between KO and WT mice. Metabolites on the right half of the scatterplots are predicted to be positively associated with each protein by MR, whereas metabolites on the left half are predicted to be inversely associated with each protein. Similarly, metabolites on the top half of the scatterplots were higher in KO vs WT animals, whereas metabolites on the bottom half were lower in KO vs. WT animals. The northwest and southeast quadrants of each scatter plot are shaded to depict consistent directionality between the MR predictions and KO experiments. The levels of each metabolite that were significantly different in KO vs. WT animals ($p \leq 0.05$) are shown as fold changes compared to WT animals (b, d, f). * indicates $P<0.05$, and ** indicates $P<0.01$ by students two tailed t-test.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and virus strains		
Biological samples		
Chemicals, peptides, and recombinant proteins		
Critical commercial assays		
Deposited data		
Experimental models: Cell lines		
Experimental models: Organisms/strains		
B6.129P2-Apoetm1Unc/J	Jackson Labs	RRID:IMSR_JAX:002052
B6.129S1-Cd36tm1Mfe/J	Jackson Labs	RRID:IMSR_JAX:019006
C57BL/6N-Acy1em1(IMPC)J/Mmucd	The Knockout Mouse Project	RRID:MMRRC_046467-UCD
C57BL/6J	Jackson Labs	RRID:IMSR_JAX:000664
Oligonucleotides		
Recombinant DNA		
Software and algorithms		
Original code to perform protein-metabolite association studies	This paper	https://zenodo.org/record/7930898

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
protein-metabolite association data	This paper	https://zenodo.org/record/7930898