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Untargeted Metabolome- and Transcriptome-Wide Association Study Suggests Causal Genes Modulating Metabolite Concentrations in Urine

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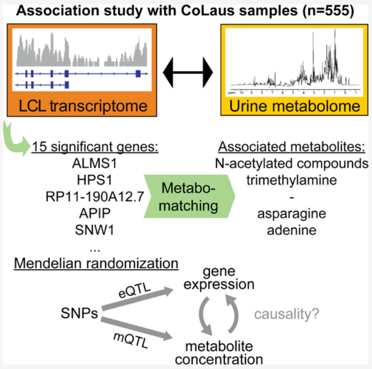
ABSTRACT: Gene products can affect the concentrations of small molecules (aka "metabolites"), and conversely, some metabolites can modulate the concentrations of gene transcripts. While many specific instances of this interplay have been revealed, a global approach to systematically uncover human gene-metabolite interactions is still lacking. We performed a metabolome- and transcriptome-wide association study to identify genes influencing the human metabolome using untargeted metabolome features, extracted from ^{1}H nuclear magnetic resonance spectroscopy (NMR) of urine samples, and gene expression levels, quantified from RNA-Seq of lymphoblastoid cell lines (LCL) from 555 healthy individuals. We identified 20 study-wide significant associations corresponding to 15 genes, of which 5 associations (with 2 genes) were confirmed with follow-up NMR data. Using metabomatching, we identified the metabolites corresponding to metabolome features associated with the genes, namely, N-acetylated compounds with *ALMS1* and trimethylamine (TMA) with *HPS1*. Finally, Mendelian randomization analysis supported a potential causal link between the expression of genes in both the *ALMS1*- and *HPS1*-loci and their associated metabolite concentrations. In the case of *HPS1*, we additionally observed that TMA concentration likely exhibits a reverse causal effect on *HPS1* expression levels, indicating a negative feedback loop. Our study highlights how the integration of metabolomics, gene expression, and genetic data can pinpoint causal genes modulating metabolite concentrations.



KEYWORDS: *transcriptomics, metabolomics, genome-wide association study, ALMS1, NAT8, HPS1, PYROXD2, N-acetylated compounds, trimethylamine*

■ INTRODUCTION

Genome-wide association studies (GWAS) have identified thousands of common variants that are associated with complex traits,[1] but the regulatory mechanisms behind these associations mostly remain poorly understood. Pinpointing causal variants is difficult since the lead variants associated with a trait are often in high linkage disequilibrium (LD) with other variants in the same region with only a slightly lower association signal. Such associated LD blocks typically contain several genes or functional elements, preventing the accurate identification of causal genes. Furthermore, some trait associated variants fall into intergenic regions of the genome with no obvious functional role at all.

A number of studies reported that trait associated genetic variants are significantly enriched in expression quantitative trait loci (eQTLs), suggesting that many trait associated variants affect the phenotype by altering gene expression.[2−5] There is also a growing body of literature highlighting the more pronounced effects of genetic variants on molecular traits compared to phenotypic traits.[6−9] This is not surprising since molecular traits representing fundamental biological processes such as gene expression and metabolism are intermediates in the causal chain from genotype to phenotype.

With high-throughput measurements becoming more accessible and widespread, integration of molecular traits into association studies has become a central challenge in the field. Such synthesis allows investigating the interplay between different organizational layers of a biological system. Despite metabolism and gene expression regulation both being fundamental biological processes that are commonly studied as molecular phenotypes, there are very few studies in humans that focus on the interplay between them. Several studies investigated the relationship between serum metabolites and whole blood gene expression in humans,[10−12] but to the best of our knowledge, no transcriptome- and metabolome-wide

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association study has been performed using urine metabolome data of healthy human subjects. Most metabolome- and genome-wide association studies (mGWAS) reporting metabolite quantitative trait loci (mQTL) use targeted approaches where the concentrations of a limited number of metabolites are estimated from the metabolome data generated by mass spectrometry or NMR spectroscopy. This targeted approach is limited to the number of known quantifiable metabolites in the biofluid under study.

In the current study, we adopted an untargeted approach, making use of the entire metabolomic data captured by binned urine ^{1}H NMR spectra as our molecular traits. We use RNAseq data from lymphoblastoid cell lines (LCLs). LCLs have been widely used in genomic studies and proven their usefulness as surrogates of primary tissues for studying both gene expression variation among individuals and the genetic architecture underlying regulatory variation of gene expression.[13−16] While LCLs partially reflect the genetic variance of gene expression in primary tissues affecting the urine metabolome, they do have the advantage of not being influenced by immediate environmental factors such as recent changes in the diet or exposure to drugs. Our transcriptome and metabolome data was taken from 555 healthy individuals that were part of the Cohort Lausannoise (CoLaus).[17] In addition, using a different NMR platform, metabolomic profiles were generated for a subset of 315 individuals from follow-up urine samples taken after 5 years. We identified several associations between expression levels and urine metabolome features that were partly validated with the follow-up data, allowing us to refine previous links between the corresponding genes and metabolites.

■ MATERIALS AND METHODS

Study Samples

CoLaus (Cohorte Lausannoise) is a population-based cross-sectional study of 6188 healthy participants residing in Lausanne, Switzerland.[17] Recruitment to the cohort was done on the basis of a simple, non-stratified random selection of the entire Lausanne population aged 35 to 75 in 2003. While all participants were genotyped, a random subset of 1000 participants was selected for NMR spectroscopy of baseline urine samples. For a random subset of 555 participants (limited by available funding and sample preparation success rate), transcriptome analysis by RNA sequencing of lymphoblastoid cells was performed (see below). The mean age of these subjects with transcriptome and metabolome profiles was 55 (min = 35, max = 75) and 53% of them were women. For a further subset of 315 participants NMR spectroscopy was performed on urine samples taken at a follow-up 5 years after the baseline.

Metabolomics Data

Baseline urinary metabolic profiles were generated using one-dimensional proton nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were acquired at 300 K on a Bruker 16.4 T Avance II 700 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) using a standard ^{1}H detection pulse sequence with water suppression. The spectra were referenced to the TSP signal and phase and baseline corrected. We binned the spectra into chemical shift increments of 0.005 ppm, obtaining metabolome profiles of 2200 metabolome features, of which 1276 remain after filtering for missing values.[18] Lastly, the data set was log10-transformed and standardised first across features (thereby normalizing the concentration of each sample) then across samples (thereby making intensities comparable). We used the z-score as a standardization method. We decided to use this statistical normalization approach, as opposed to normalizing to maximum or total creatinine levels, because it resulted in similar associations with transcriptome data, but with higher significance.

The follow-up data were acquired with an Avance III HD 600 NMR spectrometer. Spectra were referenced to the TSP signal and phase and baseline corrected. We binned the chemical shifts into 0.005 ppm bins. After removing water and urea spectral regions (4.55−5.00 ppm and 5.5−6.1 ppm), the data set was log10-transformed and standardised as described above. Our final metabolic data set includes 1289 features.

PCA plots for baseline and follow-up metabolomics data and a comparison between the baseline and follow-up urine NMR data are shown in Figure S1.

Gene Expression Data

Total RNA was extracted from Epstein−Barr-virus-transformed lymphoblastoid cell lines (LCLs) by following the Illumina TruSeq v2 RNA Sample Preparation protocol (Illumina, Inc., San Diego, CA) by the Department of Genetic Medicine and Development at the University of Geneva. Next, mRNA sequencing was performed on the Illumina HiSeq2000 platform producing 49 bp paired-end reads. On average, 17.5 million RNA-Seq reads were sequenced per sample, and its distribution is shown in Figure S2. Paired-end reads were mapped to human genome assembly GRCh37 (hg19) with GEMTools using GENCODE v15 as gene annotation.[19] The reads were then filtered for concordant orientation of the two ends and a minimum quality score of 150 while allowing 5 mismatches at both ends. Gene level read counts were quantified with an in-house script. This resulted in expression profiles of 45,470 genes for 555 individuals, which were quantified as RPKM (reads per kilobase per million reads) values. The number of genes with RPKM > 0 was on average 23,000 in each sample (Figure S2). We transformed RPKM values by applying log-transformation [$\log_2(1 + \text{RPKM})$] and then standardization (i.e., z-scoring) across samples to make genes comparable. For our analysis, we excluded genes on sex chromosomes as well as on the mitochondrial chromosome, resulting in 43,614 genes to use in the association analysis.

Genotypic Data

Genotyping was performed by using the Affymetrix GeneChip Human Mapping 500 K array set, and the imputation was carried out for HapMap II SNPs. Further details of genotype calling and the imputation can be found in Rueedi et al.[18]

Association Analysis

All statistical analyses were performed using Matlab.[20] Urine metabolome features were transformed to be normally distributed, conserving their rank in order to remove strong outlier effects.

We used a linear regression model for each pair of (transformed) metabolome feature as the response variable and gene expression level as the explanatory variable. The model also included the following common confounding factors: age, sex, the first four principal components of the genotypic data (correcting for population stratification), and the first 50 principal components of the gene expression data (correcting for potential batch effects). We tested 1276 metabolome features for association with the expression of 19,123 protein coding and 24,491 non-coding genes. We decided to not apply any a priori exclusion criteria to remove genes from the analysis, as any such criterion would be arbitrary. Instead, for genes with significant associations, we evaluated the distribution of RPKM values to ensure close to normal distribution for accurate regression estimations. In particular, we kept genes if the maximum RPKM was larger than 1 or if RPKM values were larger than 0 for ≥5% of samples.

We applied a nominal Bonferroni threshold for multiple testing $p_{\max} = 0.05/(125 \times 1109) = 3.6 \times 10^{-7}$ by taking into account the effective number of independent tests which we estimated to be 125 for metabolome features and 1109 for genes (i.e., the number of principal components explaining more than 95% of the data, as proposed by Gao et al.[21]). Associations with p values below $p_{\max}$ were considered significant.

Metabomatching

Metabomatching is a method to identify metabolites underlying associations of SNPs with metabolome features.[18,22] It compares the association profile of a given variable with all metabolome features across the full ppm range, so-called pseudospectrum, with NMR spectra of pure metabolites available in public databases such as HMDB.[23]

While metabomatching was originally developed to use SNP-metabolome associations, we recently showed that it can also identify metabolites based on co-varying features in NMR data.[24] In the present study, we use metabomatching to identify metabolites that are associated with gene expression.

Mendelian Randomization

We performed Mendelian randomization (MR) analysis[25,26] to assess the causal relationship between gene expression and metabolite concentration, using SNPs as instrumental variables (IVs), gene expression as exposure, and metabolome features as the outcome (or vice versa in the case of testing for reverse causality). For the MR analysis, we used mQTL and eQTL summary statistics from studies with greater statistical power, i.e., from untargeted mQTL study by Raffler et al.[27] (n = 3861) and from the largest blood eQTL database eQTLGen Consortium[28] (n = 31,684).

Causal effects were estimated by using the Wald method where the effect of a given genetic variant on the outcome is divided by the effect of the same genetic variant on the exposure.[29] Next, ratio estimates from different instruments (SNPs) were combined using the inverse variance weighted method (IVW) to calculate the causal estimate.[30]

We selected significant SNPs from relevant eQTL studies as our IVs. To detect independent SNPs, we used a stepwise pruning approach where we first selected the strongest lead eQTL and then pruned the rest of the SNPs in a stepwise manner if they were correlated with the lead SNP ($r^2 > 0.2$). We repeated the pruning process with the next available SNP until there were no SNPs left to prune. We used Cochran's Q test to determine heterogeneity among the candidate IVs.[31] Heterogeneous SNPs were removed in a stepwise manner from the model until the model did not show any more signs of heterogeneity (Cochran's Q statistic p value >0.05/# of original instruments). We applied four different meta-analysis (MR inference) methods to evaluate the significance of the causal estimates: Inverse variance weighted, maximum-likelihood, weighted median, and MR-Egger. The latter two methods are robust methods that have more relaxed MR assumptions, can tolerate the violation of the exclusion-restriction assumption for some instruments, and are thus less sensitive to heterogeneity among IVs. When using these robust MR methods, we did not remove any heterogeneous instruments. For all MR analysis, we used the Mendelian randomization package implemented in R.[32]

RESULTS

Association Analysis Identifies 20 Significant Metabolome- and Transcriptome-Wide Associations

We performed an untargeted metabolome- and transcriptome-wide association study by pairwise linear regression of each of 1276 metabolome features (as response variable) onto each of log-transformed expression levels of each of 43,614 genes (as explanatory variable) quantified in 555 healthy subjects (see Materials and Methods). Metabolome features resulted from binning raw urinary NMR spectra with a bin-size of 0.005 ppm, and rank-normalizing each bin passing quality control (see Materials and Methods). Gene expression levels were quantified as RPKM from RNAseq on lymphoblastoid cell lines derived for each subject (see Materials and Methods).

The QQ-plot of all pairwise associations (Figure 1) is well calibrated, with four highly significant associations (FDR < 0.05) (involving the *ALMS1* and *HPS1* genes). Applying an adjusted Bonferroni threshold of 3.6×10^{-7} to account for the effective number of independent variables (see Materials and Methods), we identified 20 additional less significant ("suggestive") feature-gene associations. These 24 association pairs involved 19 unique genes and 21 unique features. As we did not apply any a priori exclusion criteria to remove genes from the analysis, we inspected the expression value distributions of these 19 significant genes in order to identify cases in which the small p value may be due to a problematic distribution of the expression values (see Materials and Methods). This resulted in four genes to be removed, corresponding to four significant association pairs (Figure S3). The remaining 20 gene-feature associations are listed in Table 1 and their distributions are presented in Figure S4.

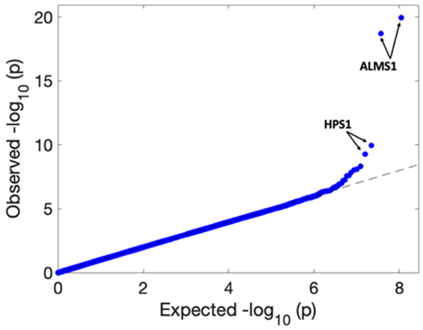

Figure 1. QQ-plot of $-\log_{10}$ (p values) of metabolome- and transcriptome-wide association analysis. The highly significant associations (FDR < 0.05) with *ALMS1* expression are ranked 1st and 2nd and with *HPS1* expression 3rd and 4th.

Identification of Metabolites Corresponding to Metabolome Features Associated with Gene Expression

To identify metabolites underlying these significant associations between gene expression levels and metabolome features, we used metabomatching,[18,22] which has been previously

Table 1. 20 Study-Wide Significant Associations from Metabolome- and Transcriptome-Wide Association Analysis[a]

genes			metabolite	association		published as mGWAS
ensembl gene ID	Chr	gene symbol	feature(s)	effect size	p value	body fluid
ENSG00000116127	2	ALMS1	2.0375, 2.0325, 2.0275, 2.0425	0.72, 0.69, 0.45, 0.41	1.1×10^{-20}, 2.0×10^{-19}, 7.8×10^{-09}, 1.2×10^{-07}	serum,[9,33] urine[27,34]
ENSG00000107521	10	HPS1	2.8575, 2.8725	−0.38, −0.37	1.1×10^{-10}, 5.6×10^{-10}	serum,[9,33] urine[27,34]
ENSG00000149089	11	APIP	2.7925	−0.33	4.7×10^{-09}	serum,[9,33] urine[27]
ENSG00000256029	1	RP11-190A12.7	3.0925	0.26	9.7×10^{-09}	serum[9]
ENSG00000100603	14	SNW1	8.1275	−0.38	1.5×10^{-08}	serum[33]
ENSG00000163016	2	ALMS1P	2.0325, 2.0375	0.27, 0.27	2.5×10^{-08}, 2.7×10^{-08}	serum,[9,33] urine[27]
ENSG00000219257	6	RP11-14I4.2	2.3275	−0.25	5.7×10^{-08}	
ENSG00000259357	1	RP11-316M1.12	7.7875	0.33	6.1×10^{-08}	
ENSG00000163520	3	FBLN2	5.4375	0.29	9.5×10^{-08}	serum[9,33]
ENSG00000226430	8	USP17L7	2.7075	−0.24	1.8×10^{-07}	
ENSG00000219355	12	RPL31P52	2.8675	−0.23	2.1×10^{-07}	
ENSG00000266795	17	RP11-744K17.9	7.2725	0.26	2.2×10^{-07}	serum[9]
ENSG00000150593	10	PDCD4	5.4075	0.42	2.2×10^{-07}	serum,[9,33] urine[27]
ENSG00000266805	18	RP11-61L19.1	5.3525	0.24	2.7×10^{-07}	
ENSG00000254396	9	RP11-56F10.3	3.0925	0.23	3.6×10^{-07}	

[a] 20 study-wide significant associations involving 15 unique genes and 17 unique features. Associations are grouped by genes and sorted by the lowest association p value for each gene.

established as an effective tool for prioritizing candidate metabolites underlying profiles of SNP-metabolome feature associations, so-called pseudospectra.[18,27] In this study, we used profiles of metabolome features that were associated significantly with one of the 15 identified genes as input to metabomatching.

We found that the pseudospectrum of the strongest associating gene, *ALMS1,* consists of four neighboring features at 2.0375 ppm (p value = 1×10^{-20}), 2.0325 ppm (p value = 2×10^{-19}), 2.0275 ppm (p value = 8×10^{-9}), and 2.0425 ppm (p value = 1×10^{-7}). A peak in this region is a typical feature of N-acetylated compounds (NACs) in their ^{1}H NMR spectra[35] but is also found in NMR spectra of other compounds (see Figure 2A). Although NAC was not ranked first by metabomatching, it is the only compound with the strongest peak at 2.0375 ppm, and the association with *ALMS1* expression at the strongest peaks of other compounds is not very significant. We therefore believe that *ALMS1* is likely associated with one or several NACs. To investigate if we could pinpoint a specific NAC, we built a library consisting of all NAC proton NMR spectra from HMDB and the Biological Magnetic Resonance Data Bank (BMRB). Metabomatching gave similar scores to many of these compounds (Figure S5). For the first ranked compound, acetylglycine, we would have expected a strong association of *ALMS1* expression with its other peak at ∼3.7 ppm, which is not the case. In fact, the secondary strong association signal in the pseudospectrum of *ALMS1* is at ∼1.7 ppm and this matches a resonance in the NMR spectra of *N*(alpha)-acetyl-dl-ornithine and *N*(alpha)-acetyllysine, making these two compounds likely candidates. *N*-acetyl-L-aspartate (NAA) is a further possible candidate due to matches of its NMR features at around 7.92 and 2.71 ppm (Figure S5) with an association signal at these positions in the *ALMS1* pseudospectrum (p value = 4×10^{-3} and 4×10^{-2}, respectively).

The pseudospectrum of *ALMS1P* (*ALMS1* pseudogene) similarly points to NAC (including *N*-acetylneuraminate and NAA) as likely matching compounds (Figure S6), although with less significant association p values (see Table 1).

The reference spectrum of NAA in the Urinary Metabolome Database (UMDB) that we used for metabomatching was recorded in water. In order to verify that the peaks of this spectrum are comparable to those of NAA in urine, we spiked NAA into pooled urine samples from our collection at a concentration of 10 mM and recorded its ^{1}H NMR spectrum. Inspecting the 5 multiplet regions of NAA, we concluded that the NAA peak positions are very similar in both solvents (Figure S7).

Two associations, which are the third and fourth strongest in this study, are between *HPS1* expression (second strongest associating gene) and two neighboring metabolome features at 2.8575 ppm (p value = 1×10^{-10}) and 2.8725 ppm (p value = 6×10^{-10}), respectively. This pseudospectrum matched well with the trimethylamine (TMA) NMR spectrum (Figure 2B). Among the top three metabolites suggested by metabomatching, trimethylamine (TMA) is the most plausible metabolite driving the association pattern because all the other metabolites have secondary peaks for which we have no significant association signal, while TMA has just a single peak in the 2.86 ppm region.

For the third strongest associating gene, *APIP*, metabomatching suggested asparagine as a metabolite (Figure S8), and the pseudospectrum of *SNW1* (5th strongest associating gene) matched well with the single peak compound adenine (Figure S9). For the remaining 10 significantly associating genes, which were associated with only one metabolome feature, metabomatching was not able to identify a corresponding metabolite.

Validation of Significant Metabolome−Transcriptome Associations Using Follow-up Urine NMR Data

To the best of our knowledge, there is no independent data set with urine NMR spectra and gene expression data of LCLs of sufficient sample size for proper out-of-sample replication of our results. However, we generated additional NMR spectra from urine samples collected from a subset of 315 CoLaus subjects in a follow-up study conducted 5 years after the baseline data collection. We note that the follow-up NMR data

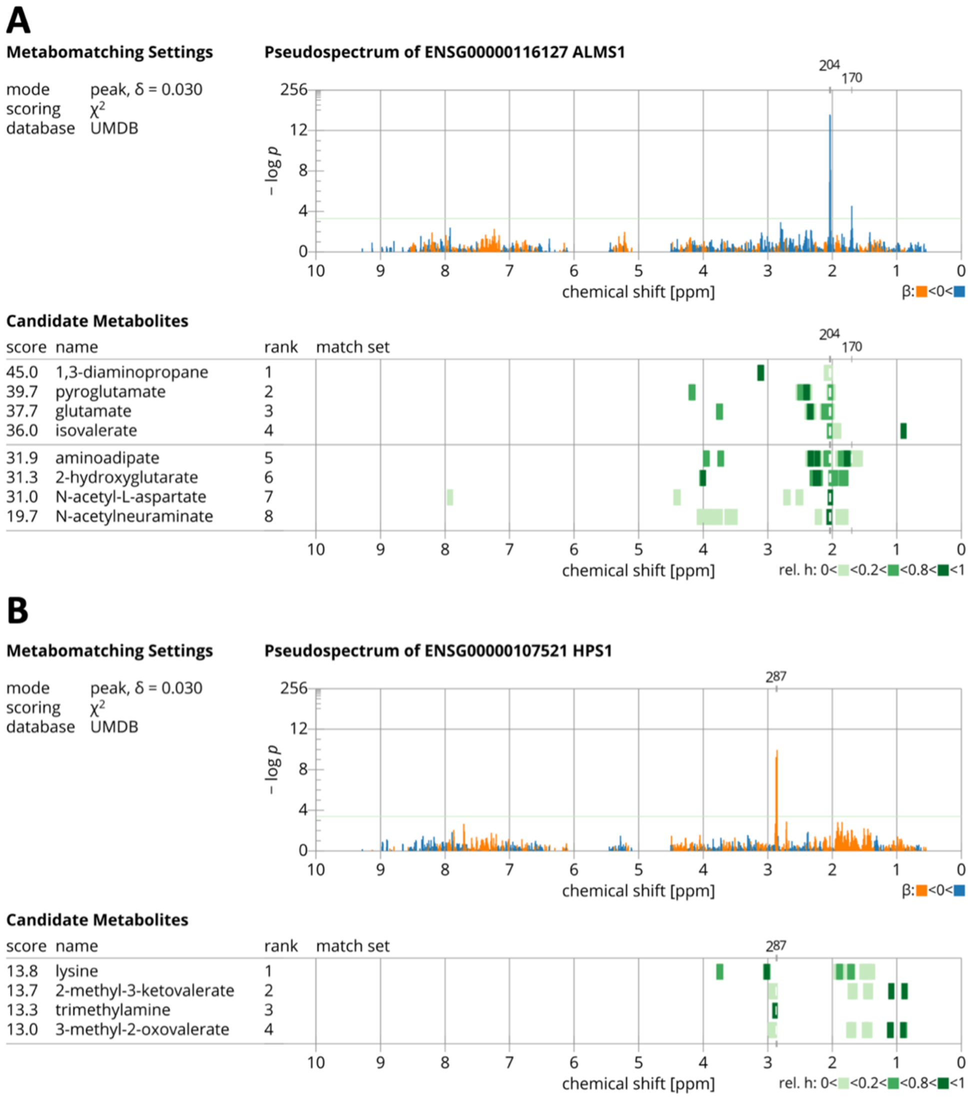

Figure 2. Metabomatching[22] results for pseudospectra derived from gene expression - metabolome feature associations for *ALMS1* (A) and *HPS1* (B). Upper panels show the features in each pseudospectrum, color-coded according to the direction of the effect (positive in blue and negative in orange). Lower panels show the highest ranking candidate metabolites with their reference NMR spectra (color coded to indicate their relative peak intensities). Leading features allowing metabolite identification are in (A) at 2.04 ppm, which matches well with the highest intensity peak of the NAA spectrum and in (B) at 2.87 ppm, which matches well with the TMA singlet.

are not independent from the baseline data, yet they were obtained from physically different samples collected at a significantly later time and processed with a different NMR spectrometer and facility. As for the expression data, we only have those from LCLs derived from blood taken at the baseline, so we could only test whether the associations we observed between baseline metabolomics and baseline transcriptomics measurements would persist as associations between follow-up metabolomics and baseline transcriptomics data.

As baseline and follow-up urine NMR data were each processed and binned individually, the features did not correspond one-to-one between the studies (see Materials and Methods). Thus, to validate the association of genes with relevant features, we tested the two nearest features to every top feature associated with a gene in the baseline data set. We used the Benjamini−Hochberg (BH) procedure[36] to detect significant replications; more specifically, we considered replications validated if the p value was smaller than 0.05/(2 features x rank of the association in the discovery) for any one of the two tested features. In the follow-up, both *ALMS1* and *HPS1* gene expression levels were associated significantly with features corresponding to those of the discovery study (Table S1). The third most significantly associating gene with the baseline metabolome data, *APIP*, yielded an association in the follow-up data with p value = 9.8×10^{-3}, which is just above its BH threshold of 0.005. All other genes did not show any significant association with representative features in the follow-up study.

mGWAS for NAC and TMA Indicates Numerous Significant SNPs in the *ALMS1* and *HPS1* Gene Loci

To get a broader overview of the genetic influences on metabolome features in the NAC and TMA NMR peak regions, we performed an untargeted metabolome- and genome-wide association study using data from 826 individuals of the CoLaus cohort, for whom baseline urinary NMR spectra were available (similar to Rueedi et al.[18]). SNPs that are significantly associated with the NAC metabolome feature at

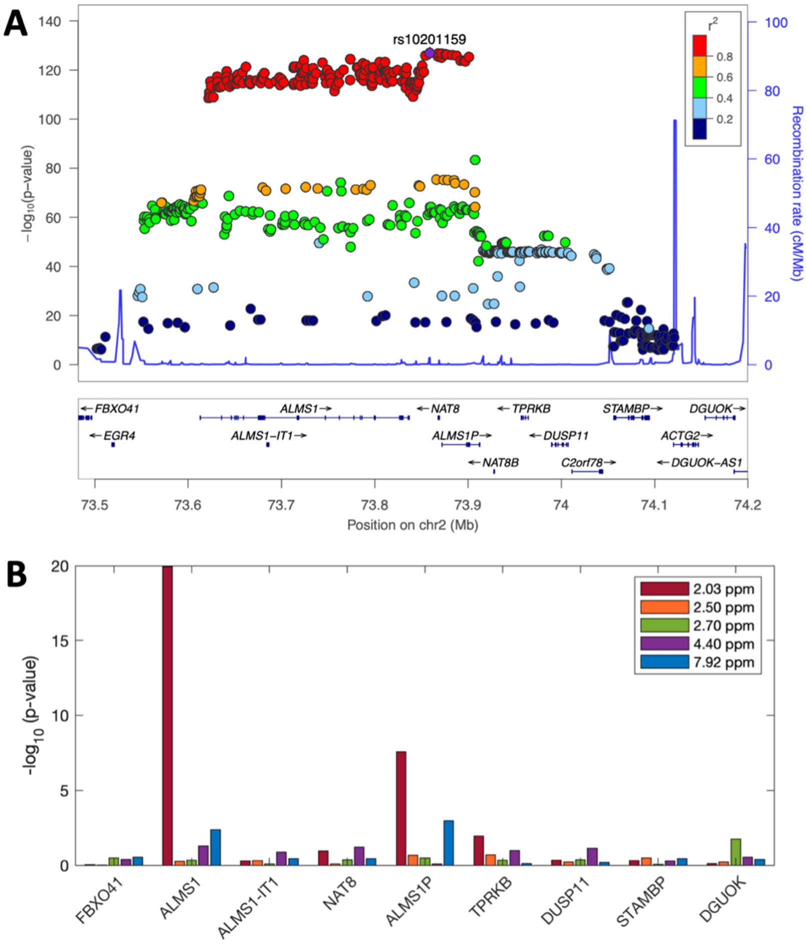

Figure 3. SNP - metabolome feature and SNP - gene expression associations in *ALMS1*/*NAT8* locus. (A) LocusZoom plot for *ALMS1*/*NAT8* locus, where the SNPs are associated with metabolome feature at 2.0375 ppm, LD colored with respect to lead mQTL. (B) Bar plot shows $-\log_{10}$ transformed p values from associating expression values of nine genes in the locus with the five NAA features.

2.0375 ppm are correlated with each other ($r^2 > 0.8$) and lie within an LD block containing nine genes, including *ALMS1*, *ALMS1-IT1*, *NAT8*, and *ALMS1P* (Figure 3A). Out of these nine genes, *ALMS1* and *ALMS1P* have the most significant association results with the 2.0375 ppm feature and with the NAA feature at 7.9225 ppm (Figure 3B). However, we note that the expression levels in LCLs differ between genes; in particular, *NAT8* has RPKM = 0 in most samples, which could impact its association results. Similarly, SNPs that associate with the TMA metabolome feature at 2.8725 ppm are also highly correlated and lie in a locus surrounding the *HPS1*/*PYROXD2* genes (Figure 4A). Even though the SNPs with the most significant association with feature 2.8725 are physically located closer to *PYROXD2* rather than *HPS1*, the expression level of *PYROXD2* does not show a significant association with this feature (Figure 4B). Notably here, *HPSE2*, *MIR1287*, and *MIR4685* are very lowly expressed (RPKM = 0 in most samples).

To further evaluate a possible regulation of NAC and TMA by other genes suggested by published mGWAS studies, we investigated the pseudospectra of these using metabomatching. In particular, we investigated genes that either were the target of an eQTL SNP that is a mQTL of NAC/TMA or were within 500 kb of *ALMS1*/*HPS1*. However, none of these candidate genes (14 for the *ALMS1*-NAC and 6 for the *HPS1*-TMA association pair) produced a pseudospectrum containing even a single nominally significant signal pointing to these metabolites (data not shown).

Inspecting published mGWAS in humans,[37] we found that the SNPs in both *ALMS1* and *HPS1* loci have been previously reported to associate with a number of metabolic traits (Table 2). The *ALMS1* locus has been associated with N-acetylated compounds, while the *HPS1* locus has been associated with various metabolites including trimethylamine and dimethylamine.[18,27,34] In mGWAS studies, the most likely candidate genes are usually inferred based on their physical proximity to the lead mQTL and, if available, their functional annotation. Indeed, published mGWAS studies were not able to distinguish between *NAT8* or *ALMS1* and *HPS1* or *PYROXD2* as genes affecting NAC and TMA, respectively. In contrast, our association study using gene expression data from LCLs clearly favors *ALMS1* and *HPS1* as the relevant genes. However, for *NAT8*, which is mostly not expressed in LCLs, conclusions on its association with NAC in functionally more relevant tissues are not possible.

A likely genetically driven relationship between *ALMS1* and NAC concentrations measured in the baseline data set, through a shared eQTL and mQTL (SNP rs7566315), is illustrated in Figure 5.

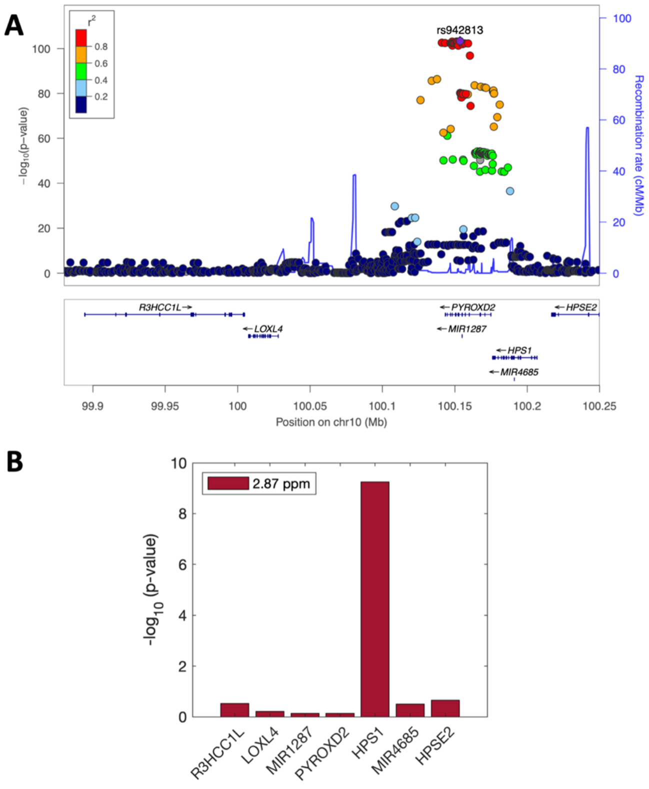

Figure 4. SNP - metabolome feature and SNP - gene expression associations in *HPS1*/*PYROXD2* locus. (A) LocusZoom plot for *HPS1*/*PYROXD2* locus, showing the association significance of SNP with the metabolome feature at 2.8725 ppm. Colors indicate the correlation (LD) to the lead QTL. (B) Bar plot shows $-\log_{10}$ transformed p values from associating expression values of seven genes in the locus with the same feature.

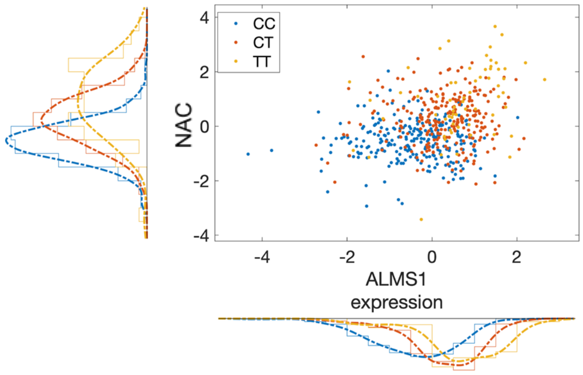

Figure 5. Scatter plot of the mQTL effect of SNP (rs7566315) on NAC and its eQTL effect on *ALMS1* gene expression. Each point represents a study sample. NAC concentration is approximated by the feature at 2.0375 ppm that is $\log_{10}$ transformed after feature- and sample-wise z-scoring (y axis). *ALMS1* expression is z-scored after $\log_2$ transforming RPKM+1 values (x axis). Color code represents the genotype of rs7566315 (legend) that is an eQTL of *ALMS1* and mQTL of NAA.

Mendelian Randomization Analysis Suggests *ALMS1* Expression in Blood and Confirms *NAT8* Expression in Other Tissues to Causally Effect NAC Concentration

To assess a causal relationship between gene expression and metabolite concentration, we performed MR analysis using SNPs as instrumental variables (IVs) selected based on summary statistics from the eQTLGen Consortium[28] and Raffler et al.[27] for eQTL and untargeted mQTL results, respectively. We investigated both the causal effect of the gene expression on the metabolite concentration and vice versa for the *ALMS1*-NAC and *HPS1*-TMA gene-metabolite pairs.

We first investigated the causal effect of the *ALMS1* gene on NAC concentration reflected by the NMR peak intensity at 2.0308 ppm.[34,44] We considered 86 SNPs that were significant eQTLs (FDR < 0.05) in eQTLGen and that were also identified as mQTLs by Raffler et al. After stepwise pruning (see Materials and Methods), 14 independent SNPs remained as candidate IVs. Next, we performed Cochran's Q test to detect heterogeneity among these SNPs and removed a further three, resulting in 11 SNPs as potentially valid IVs to use in the MR analysis (see Materials and Methods). Our causal effect estimates given by four meta-analysis methods (inverse variance weighted, weighted median, MR-Egger, maximum-likelihood; see Materials and Methods) are reported in Table 3A. All methods agree on the *ALMS1* expression level being

Table 2. Previously Reported mGWAS Results for the *ALMS1*/*NAT8* and *HPS1*/*PYROXD2* Loci[a]

reference	platform	biofluid	locus	metabolite
Nicholson et al. 2011[34]	MS + NMR	urine + plasma	ALMS1, NAT8	N-acetylated compounds
Montoliu et al. 2013[44]	NMR	urine	ALMS1	N-acetylated compounds
Rueedi et al. 2014[18]	NMR	urine	ALMS1	2.0375 (suggested as N-acetylated compounds)
Raffler et al. 2015[27]	NMR	urine	NAT8	2.031 (suggested as *N*-acetyl-L-aspartate)
Suhre et al. 2011[38]	MS	serum	NAT8	*N*-acetylornithine
Yu et al. 2014[39]	MS	serum	NAT8	*N*-acetylornithine
Shin et al. 2014[33]	MS	serum	NAT8	*N*-acetyllysine, unknown compounds
Nicholson et al. 2011[34]	MS + NMR	urine + plasma	HPS1, PYROXD2	trimethylamine (urine), dimethylamine (plasma),
Rueedi et al. 2014[18]	NMR	urine	PYROXD2	trimethylamine, unknown compound, 2.8575, 1.8025
Raffler et al. 2015[27]	NMR	urine	PYROXD2	2.854 (suggested as trimethylamine)
Raffler et al. 2013[40]	NMR	plasma	PYROXD2	2.757
Rhee et al. 2013[41]	MS	plasma	HPS1	asymmetric dimethylarginine
Krumsiek et al. 2012[42]	MS	serum	HPS1, PYROXD2	multiple compounds, unknown compounds
Hong et al. 2013[43]	MS	serum	HPS1	caprolactam
Shin et al. 2014[33]	MS	serum	PYROXD2	unknown compounds

[a]MS: mass spectrometry; numbers in metabolite section refer to NMR spectral shift positions in ppm. Reported genes are mostly based on proximity to the mQTL or based on gene function.

Table 3. MR Results for Testing a Causal Link between *ALMS1* Expression and Concentration of N-Acetylated Compounds[a]

		method	causal effect size estimate	std. error	95% CI	p value	Cochran's Q-statistic p value
A	ALMS1 → NAC	inverse variance weighted	0.967	0.061	0.847−1.087	$<2 \times 10^{-16}$	0.2323
		weighted median	1.111	0.075	0.965−1.257	$<2 \times 10^{-16}$	NA
		MR - Egger	0.994	0.092	0.812−1.175	$<2 \times 10^{-16}$	0.1776
		maximum-likelihood	0.999	0.065	0.872−1.126	$<2 \times 10^{-16}$	0.249
B	NAC → ALMS1	inverse variance weighted	−0.015	0.264	−0.532−0.502	0.955	0.7443
		weighted median	0.122	0.321	−0.507−0.751	0.704	NA
		MR - Egger	1.495	1.976	−2.377−5.368	0.449	0.7256
		maximum-likelihood	−0.015	0.266	−0.535−0.505	0.955	0.7443
C	ALMS1 → NAC (NAT8 related SNPs removed)	inverse variance weighted	0.796	0.183	0.437−1.155	$<2 \times 10^{-16}$	0.1902
		weighted median	0.668	0.242	0.193−1.142	0.006	NA
		MR - Egger	1.912	0.704	0.532−3.291	0.007	0.4144
		maximum-likelihood	0.805	0.185	0.444−1.167	$< 2 \times 10^{-16}$	0.2199

[a] MR results for testing (A) causal effect of *ALMS1* gene expression levels on N-acetylated compounds (*ALMS1* → NAC), (B) causal effect of N-acetylated compounds on *ALMS1* gene expression levels (NAC → *ALMS1*), (C) causal effect of *ALMS1* gene expression levels on N-acetylated compounds (*ALMS1* → NAC) when *NAT8*-related SNPs were removed from the instrument set.

Table 4. MR Results for Testing a Causal Link between *HPS1* Expression and TMA Concentration[a]

		method	causal effect size estimate	std. error	95% CI	p value	Cochran's Q-statistic p value
A	HPS1 → TMA	inverse variance weighted	0.266	0.094	0.082−0.450	0.005	0.0803
		weighted median	0.311	0.072	0.170−0.453	$<2 \times 10^{-16}$	NA
		MR - Egger	0.37	0.126	0.123−0.617	0.003	0.0852
		maximum-likelihood	0.267	0.094	0.083−0.452	0.004	0.0829
B	TMA → HPS1	inverse variance weighted	−0.089	0.012	−0.113 to −0.065	$<2 \times 10^{-16}$	0.0958
		weighted median	−0.09	0.011	−0.111 to −0.068	$<2 \times 10^{-16}$	NA
		MR - Egger	−0.086	0.013	−0.111 to −0.061	$<2 \times 10^{-16}$	0.0758
		maximum-likelihood	−0.09	0.012	−0.114 to −0.066	$<2 \times 10^{-16}$	0.1258
C	HPS1 → TMA (PYROXD2 related SNPs removed)	inverse variance weighted					
		weighted median	1.079	0.121	0.842−1.315	$<2 \times 10^{-16}$	NA
		MR - Egger	1.705	0.305	1.107−2.303	$<2 \times 10^{-16}$	0.5575
		maximum-likelihood					

[a] MR results for testing (A) causal effect of *HPS1* gene expression levels on TMA (*HPS1* → TMA), (B) causal effect of TMA on *HPS1* gene expression levels (TMA → *HPS1*), (C) causal effect of *HPS1* gene expression levels on TMA (*HPS1* → TMA) when *PYROXD2*-related SNPs were removed from the instrument set.

causal for NAC concentrations, indicated by p values < 2 × 10^{-16}.

As *NAT8* has a known *N*-acetyltransferase activity and was previously suggested to be involved with NAC concentration (see Table 2), we performed an additional MR analysis with *ALMS1* as the exposure where we removed the possible pleiotropic effect of the *NAT8* gene. To achieve this, we excluded 10 out of 14 original candidate IVs that were also significant eQTLs of *NAT8* or were in LD (R-squared > 0.1) with these *NAT8* eQTLs (as there were no *NAT8* eQTLs in eQTLGen, these were taken from GTEx consortium (v8), considering all available tissues[2] and using a significance cutoff of p value < 10^{-7}). Without these *NAT8*-related SNPs, the MR results remained significantly causal (Table 3C), indicating that the causal effect of *ALMS1* on NAC may not be driven by the pleiotropic effect of *NAT8*.

Finally, we performed an additional sensitivity analysis by lowering the *R*-squared threshold in the stepwise LD pruning process to 0.05, resulting in more strictly independent IVs for MR analysis. The significant causal effect of *ALMS1* on NAC persisted with more strictly independent instruments, indicated by all four meta-analysis methods (see Table S2, top panel). However, when removing *NAT8*-related eQTLs, only one meta-analysis method (weighted-median) indicated a significant causal effect of *ALMS1* on NAC (see Table S2, bottom panel). We also performed an MR analysis with *NAT8* as the exposure, but due to the low number of valid IVs (only two remained after removing two with heterogeneity), we could only use robust MR analysis methods. Both weighted median and MR-Egger indicated a significant causal effect of *NAT8* expression on NAC (beta = 0.36 and 0.63, respectively; p values < 10^{-16}). When excluding *ALMS1*-related SNPs, only two IVs remained and thus we could not test if the causal effect of *NAT8* is independent of ALMS1 expression.

For the completeness of the analysis, we also tested a causal effect of NAC on the *ALMS1* gene expression level. IVs were selected among the SNPs that were reported as significant mQTLs for the 2.03 ppm feature (p value < 1×10^{-6}) in

Raffler et al.[27] Among the cis-eQTLs of *ALMS1* from eQTLGen, we observed strong heterogeneity between their expected and observed effects. To overcome this problem, we sought to use also trans-eQTLs of *ALMS1*; however, none of the candidate IVs were measured in the trans-eQTL study of eQTLGen. As an alternative, we performed an association study between the candidate IVs and *ALMS1* gene expression level as measured for our 555 CoLaus individuals and used these trans-eQTL results. Overall, we identified 26 overlapping significant mQTLs and trans-eQTLs, corresponding to six independent SNPs. Two of the six candidate IVs were removed due to pleiotropic effects resulting in four SNPs to be used as potentially valid IVs in the MR analysis (see Materials and Methods). None of the four meta-analysis methods (Table 3B) gave a significant causal effect estimate, indicating that we have no evidence for NAC concentration causally affecting *ALMS1* gene expression.

In conclusion, our MR analysis using SNPs selected from eQTLs in blood tissue suggests a causal effect of *ALMS1* expression levels on NAC, which appears independent from *NAT8* gene expression (in various GTEx tissues). However, we also detected a significant causal effect of *NAT8* expression (in GTex tissues) on NAC concentration.

Mendelian Randomization Analysis Suggests *HPS1* as Causal Gene Modulating TMA Concentration and Indicates a Reverse Causal Effect between Both

We performed a similar MR analysis to test a causal relationship between *HPS1* gene expression and TMA concentration. As there were no targeted summary statistics for TMA concentration, we used the NMR peak intensity at 2.8541 ppm from Raffler et al.[27] as a proxy for TMA concentration. This position is in agreement with the singlet peak position range from 2.79 to 2.99 ppm, according to HMDB. Among the 77 SNPs that were reported as significant eQTLs for *HPS1* (FDR < 0.05) in eQTLGen and measured by Raffler et al., only six could be used as valid IVs that were independent and did not exhibit heterogeneity (see Materials and Methods). Causal effects estimated by four different meta-analysis methods were all significant (p value < 0.005; see Table 4A), suggesting that *HPS1* gene expression has a causal effect on TMA concentration.

We performed an additional MR analysis removing the possible pleiotropic effect of the other candidate gene in the locus, *PYROXD2,* as this gene was previously suggested to be involved in modulating TMA (see Table 2). For this, we removed from the original 77 candidate IVs 54 SNPs that were also significant eQTLs of *PYROXD2* (eQTLGen FDR < 0.05) or were in LD with these eQTLs (R-squared >0.1). Stepwise pruning with R-squared thresholds of 0.2 or 0.05 both resulted in 3 SNPs to be used in MR analysis. These 3 SNPs had heterogeneity; however, due to the low number of instruments, we could not further remove any of them. Therefore, we relied on robust MR methods. Both robust MR methods (weighted median and MR-Egger) indicated a significant causal effect of *HPS1* on TMA (p value $< 2 \times 10^{-16}$) with estimated causal effect sizes larger than 1.0 (Table 4C). This suggests that the causal effect of *HPS1* on TMA is not driven by a pleiotropic effect of *PYROXD2*. We also attempted an MR analysis testing the causal effect of *PYROXD2* on TMA. However, when *HPS1*-related SNPs were discarded from the candidate IVs, there were no SNPs left for the analysis.

Next, we performed a sensitivity analysis using an R-squared threshold of 0.05 in stepwise LD pruning, which showed a persisting causal effect of *HPS1* on TMA, when all *HPS1* eQTLs were used as candidate SNPs in the MR analysis (p value $< 2 \times 10^{-16}$ for all four meta-analysis methods; see Table S3, upper panel), and when *PYROXD2*-related eQTLs were removed from the candidate SNPs (p value $< 2 \times 10^{-16}$ for robust meta-analysis methods; see Table S3, bottom panel).

Finally, we tested the causal effect in the reverse direction, from TMA concentration to *HPS1* gene expression. From 87 significant mQTLs in Raffler et al.[27] that were also measured in eQTLGen, 18 SNPs remained to be used as IVs in the MR analysis after stepwise pruning and removing the SNPs showing heterogeneity (see Materials and Methods). All four meta-analysis methods agreed on TMA concentration being causal for *HPS1* expression (p value $< 2 \times 10^{-16}$; Table 4B). While the estimated causal effect size of *HPS1* on TMA ranged between 0.27 and 0.37 for different methods, the causal effect size of TMA on *HPS1* was around −0.09, pointing to the existence of a negative feedback loop.

■ DISCUSSION

In this study, we present a metabolome- and transcriptome-wide association study using RNA-seq from LCLs and NMR urine profiles from 555 subjects of the CoLaus cohort. This is the first study performed on untargeted urine metabolome data from healthy individuals. We identified two genes, *ALMS1* and *HPS1,* whose association with NMR features are highly significant, and 13 additional genes that are associated with metabolome features with lower but still significant p values (see Table 1). Among these 15 genes, 9 are in loci with SNPs that have been previously reported as mQTLs by mGWAS. This shows that our approach can identify likely candidates of metabolically relevant genes, despite a limited sample size.

To search for metabolite candidates underlying gene expression-metabolome feature associations, we used our metabomatching tool that ranks metabolites with known NMR peaks based on their match to a given pseudospectrum.[22] We consider NAC as the most likely compounds to be associated with *ALMS1* expression, even though NACs were not ranked top by metabomatching. Similarly, for *HPS1*, we consider TMA, which also did not rank top, as the most likely compound underlying the association with the NMR feature at ~2.86 ppm. The reason for this was in both cases that the most significant association peaks of *ALMS1* and *HPS1* matched best with the highest peaks in the reference spectra of NAC and TMA, respectively. Thus, metabomatching was very useful in prioritizing candidate metabolites, but its scoring could be improved by weighting the association signal based on the relative peak height of the reference spectrum, which currently is only visualized.

Our top hit *ALMS1* as well as the second strongest association involving *HPS1* had previously been implicated by mGWAS linking their loci to compound families. However, in both cases, the reported mQTLs were also in proximity to other genes, leaving the causal gene-metabolite association ambiguous. Specifically, the locus associated through mGWAS with N-acetylated compounds (NAC) includes two genes, *ALMS1* and *NAT8,*[18,27,34,44] and the latter seemed to be an appealing candidate due to its known N-acetyltransferase activity. Yet, our association study linked NAC concentrations to *ALMS1* and not *NAT8* expression in LCLs. This is probably due to the fact that expression of *NAT8* in LCLs is very low, so one would need to test its expression in tissues such as liver or kidney, where it is known to be highly expressed, which is however not feasible for a cohort study like *CoLaus*. A metabolic role of *ALMS1* is supported through its known role in Alström syndrome characterized by metabolic deficits[53] and kidney health disorder phenotypes.[45] Interestingly, in the mGWAS reported by Montoliu et al. using data from a Brazilian cohort, the authors observed the association between NAC and the SNPs located in *ALMS1*/*NAT8* locus with stronger SNP associations in the *ALMS1* gene rather than *NAT8*.[44] They argued that the high ethnic diversity of their study population might have been responsible for breaking down the linkage disequilibrium in the *ALMS1*/*NAT8* region of the genome, resulting in a stronger association for SNPs close to or within the *ALMS1* gene compared to other studies.

Intriguingly, NAA, a likely NAC underlying the observed association, is the second most abundant metabolite in the brain and is involved in neural signaling by serving as a source of acetate for lipid and myelin synthesis in oligodendrocytes.[46] NAA can be detected in urine in low concentrations,[47] and it has a long history of being a surrogate marker of neural health and a broad measure of cognitive performance.[48,49] Recently, it has been shown that NAA correlates with neuropsychological performance measures.[50] The signals of SNPs in *ALMS1* by GWAS with intellectual phenotypes such as a self-reported ability in mathematics[51,52] might therefore be due to its role in modulating NAA. This conjecture of course assumes that NAA levels in relevant brain tissues reflect those in urine and that the *ALMS1* expression variation and in particular its genetic component, in LCLs or blood, can serve as a proxy for brain tissue.

As for *HPS1*, our second strongest associating gene, we note that mGWAS previously associated its locus with TMA levels.[18,27,34] Yet, most of these studies, including the aforementioned mGWAS using a Brazilian cohort,[44] considered the *PYROXD2* gene, which is in the same locus, as the most likely modulator of TMA concentrations due to its known function as pyridine nucleotide-disulfide oxidoreductase. While we cannot rule out that *PYROXD2* is involved in TMA metabolism, our study finds no evidence for association of *PYROXD2* expression levels in LCLs with TMA concentration in urine, indicating that the mQTLs of this locus may act predominantly as eQTLs through *HPS1*.

In order to study the causal relationship between gene expression and metabolite concentrations, we conducted Mendelian randomization analyses. Our analyses supported a causal relationship between *ALMS1* expression in blood and NAC concentration in urine that appears independent of *NAT8* expression. Yet, our MR analysis also confirmed a causal effect of *NAT8* expression in other tissues (than LCLs or blood) on NAC concentration in urine. For *HPS1*, we observed a causal effect of its expression level in blood on TMA concentration in urine that appears independent of *PYROXD2* expression in addition to a negative feedback loop between *HPS1* expression levels and TMA concentrations. However, the causal links identified through MR analysis using mQTLs from urine and eQTLs mostly from blood may not represent very well the actual effects in the metabolically relevant tissues. Thus, based on the available data, MR analysis cannot unambiguously pinpoint the causal gene in the *ALMS1*/*NAT8*- and *HPS1*/*PYROXD2*-loci, whose expression drives the respective metabolite concentrations.

We acknowledge that our study has several limitations: First, the transcriptome data from LCLs may only poorly reflect the expression of genes in more functionally relevant cells and tissues. Furthermore, the mRNA expression levels may not correlate well with the protein concentrations of the enzymes involved in the metabolic reactions, as these are additionally shaped by translation regulation, post-translational modifications, or decay. Second, the metabolome data from urine may depend on food intake and may only provide a poor proxy for the metabolite concentrations in other relevant biosamples. Thus, relating LCL expression levels with urine metabolite concentrations might not allow detecting all regulations that occur in more functionally relevant tissues. This is likely the case for *NAT8*, which is barely expressed in LCLs but has high expression levels in, e.g., kidney and liver. Finally, our study is limited by the sample size of studied CoLaus data and by the available eQTLs and mQTLs, and the LD reference panel, used for MR. More highly powered studies are needed to provide a more definite answer on some casualties.

Nevertheless, our study shows a proof of concept for how global association of sets of two or more distinct molecular traits observed in the same cohort can facilitate new discoveries of how different molecular entities affect each other. Such analyses, when applied to sizable datasets, will be instrumental in unravelling the complex regulatory relationships underlying human metabolism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00585.

Figure S1: Principal component analysis of baseline and follow-up metabolomics data; Figure S2: Overview of RNA-Seq read count and quantifiable genes in 555 individuals; Figure S3: Scatter plots of removed associations; Figure S4: Scatter plots of 20 study-wide significant metabolome feature - gene expression associations; Figure S5: Metabomatching figure showing the pseudospectrum derived from *ALMS1* gene expression - metabolome features associations; Figure S6: Metabomatching figure showing the pseudospectrum derived from *ALMS1P* gene expression - metabolome features associations; Figure S7: NMR profiles of 3 different spike-in experiments; Figure S8: Metabomatching figure showing the pseudospectrum derived from *APIP* gene expression - metabolome feature associations; Figure S9: Metabomatching figure showing the pseudospectrum derived from *SNW1* gene expression - metabolome feature associations; Table S1: Validation of all associations discovered in CoLaus baseline; Table S2: MR results of testing causal effect of *ALMS1* gene expression levels on N-acetylated compounds, using *R*-squared threshold of 0.05; Table S3: MR results of testing causal effect of *HPS1* gene expression levels on trimethylamine, using *R*-squared threshold of 0.05 (PDF)

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Author Contributions

R.S.F., R.R., and S.B. designed the project. R.S.F. carried out the computational analysis and prepared the results, with help from A.B. B.K. and R.R. provided data and feedback on the metabolomics analysis. Z.K. designed and supervised the MR analysis. All authors discussed the results and provided feedback on the manuscript that was written by R.S.F., B.K., A.B., and S.B.

Notes

The authors declare no competing financial interest.

Processed transcriptome and metabolome data are accessible at Zenodo (www.zenodo.org, doi:10.5281/zenodo.5106119). This data set includes RPKM levels in LCLs for 45,484 genes and 555 individuals, binned and normalized NMR peak intensities for 1276 bins in 555 individuals from baseline urine samples, and 1289 bins in 315 individuals from follow-up urine samples.

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