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A network-based conditional genetic association analysis of the human metabolome

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Abstract:	Background: Genome-wide association studies have identified hundreds of loci that influence a wide variety of complex human traits; however, little is known regarding the biological mechanism of action of these loci. The recent accumulation of functional genomics ("omics"), including metabolomics data, has created new opportunities for studying the functional role of specific changes in the genome. Functional genomic data are characterized by their high dimensionality, the presence of (strong) statistical dependency between traits, and—potentially—complex genetic control. Therefore, the analysis of such data requires specific statistical genetics methods. Results: To facilitate our understanding of the genetic control of omics phenotypes, we propose a trait-centered, network-based conditional genetic association (cGAS) approach for identifying the direct effects of genetic variants on omics-based traits. For each trait of interest, we selected from a biological network a set of other traits to be used as covariates in the cGAS. The network can be reconstructed either from biological pathway databases or directly from the data, using a Gaussian Graphical Model applied to the metabolome. We derived mathematical expressions which allow comparison of the power of univariate analyses with conditional genetic association analyses. We then tested our approach using data from a population-based KORA study (n=1784 subjects, 1.7 million SNPs) with measured data for 151 metabolites. Conclusions: We found that compared to single-trait analysis, performing a genetic association analysis that includes biologically relevant covariates increases the power of the analysis by providing more accurate estimates of genetic effects; however, analysis can either gain additional power or even lose power, depending on specific pleiotropic scenarios, for which we provide empirical examples. We also show the importance of properly selecting sets of traits to be entered in the multivariate analysis. In the context of analyzed metabolomics data, the knowledge-based network approach has increased power. Nevertheless, we believe that our analysis shows that neither a prior-knowledge-only approach nor a phenotypic-data-only approach is optimal, and we discuss possibilities for improvement.
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A network-based conditional genetic association analysis of the human metabolome

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Abstract

Background: Genome-wide association studies have identified hundreds of loci that influence a wide variety of complex human traits; however, little is known regarding the biological mechanism of action of these loci. The recent accumulation of functional genomics ("omics"), including metabolomics data, has created new opportunities for studying the functional role of specific changes in the genome. Functional genomic data are characterized by their high dimensionality, the presence of (strong) statistical dependency between traits, and—potentially—complex genetic control. Therefore, the analysis of such data requires specific statistical genetics methods.

Results: To facilitate our understanding of the genetic control of omics phenotypes, we propose a trait-centered, network-based conditional genetic association (cGAS) approach for identifying the direct effects of genetic variants on omics-based traits. For each trait of interest, we selected from a biological network a set of other traits to be used as covariates in the cGAS. The network can be reconstructed either from biological pathway databases or directly from the data, using a Gaussian Graphical Model applied to the metabolome. We derived mathematical expressions which allow comparison of the power of univariate analyses with conditional genetic association analyses. We then tested our approach using data from a population-based KORA study (n=1784 subjects, 1.7 million SNPs) with measured data for 151 metabolites.

Conclusions: We found that compared to single-trait analysis, performing a genetic association analysis that includes biologically relevant covariates increases the power of the analysis by providing more accurate estimates of genetic effects; however, analysis can either gain additional power or even lose power, depending on specific pleiotropic scenarios, for which we provide empirical examples. We also show the importance of properly selecting sets of traits to be entered in the multivariate analysis. In the context of analyzed metabolomics data, the knowledge-based network approach has increased power. Nevertheless, we believe that our analysis shows that neither a prior-knowledge-only approach nor a phenotypic-data-only approach is optimal, and we discuss possibilities for improvement.

Short abstract

We propose a trait-centric network-based conditional approach for performing a genetic association analysis of multivariate omics phenotypes. This approach can incorporate existing biological knowledge regarding biological pathways obtained from external sources and is designed to specifically test for direct genetic effects. We applied this approach to existing metabolomics data and found that it increases power by having increased accuracy of genetic effect estimates in the presence of specific "counterintuitive" pleiotropic scenarios in which genetically induced and residual covariance are opposite. We provide examples of different pleiotropic scenarios, and we discuss possible additional applications for this approach.

Background

Genome-wide association studies (GWASs) are a highly popular method for identifying alleles that affect complex traits in humans, including the risk of common diseases. In the past decade, GWASs have enabled the identification of thousands of loci, significantly increasing our understanding of the genetic basis underlying the control of complex human traits [1]. On the other hand, this has had only a limited impact on the development of biomarkers and therapeutic agents; in most cases, any association found using GWAS approach can only serve as a starting point for future research, rather than providing a direct answer to the question of the genetic region's precise biological function. The recent accumulation of functional genomics (or "omics" for short) data—including information regarding the levels of gene expression (the transcriptome), metabolites (the metabolome), proteins (the proteome), and glycosylation (the glycome)—can provide new insight into the functional role of specific changes in the genome [2,3].

Metabolomics is an emerging field that has been studied extensively in the past decade. A number of GWASs of metabolites have been performed using various platforms [4–8], revealing literally dozens of loci associated with variations in various lipid species, amino acids, and other small molecules. Linking the variants that underlie these variations in metabolomics with various diseases can provide functional insight into the many disease-related associations that were reported in previous studies, including cardiovascular and kidney disease, type 2 diabetes, cancer, gout, venous thromboembolism, and Crohn's disease [5].

However, analyzing metabolomics data requires specialized statistical methods due to their characteristically high dimensionality and the presence of statistical dependencies that reflect biological relationships between different variables. Conventional univariate GWAS (uGAS) approaches ignore any possible dependencies between different omics traits, which can confound the biological interpretation of the results and may lead to a loss of statistical power. On the other hand, utilizing multivariate phenotype information increases the statistical power of the association tests compared to univariate analysis [9–12]. Despite a large number of methodological studies, however, only a few empirical multivariate GWASs have been published using data for humans. We recently demonstrated [13] that using a multivariate analysis can substantially increase the power of locus identification in the context of human *N*-glycomics; indeed, not only did our multivariate analysis double the number of loci identified in the analysis sample, but also all five novel loci were strongly replicated. With respect to metabolomics, Inouye et al. [6] performed a multivariate GWAS on 130 metabolites (grouped in 11 sets) measured in approximately 6600 individuals. They found that multivariate analysis doubled the number of loci detected in this sample; seven of these additional loci discovered were novel loci that had not been identified

previously in other GWAS analyses of related traits. While no replication of novel loci was performed by Inouye et al., we compared the authors' results with a recently published univariate GWAS of metabolomics derived from a cohort containing nearly 25,000 individuals [8]. We found that three of the seven SNPs reported by Inouye et al. have a *p*-value $< 5 \times 10^{-11}$ for at least one metabolite (i.e., are significant at the genome-wide level after Bonferroni correction for 130 analyses). These findings provide empirical evidence supporting the value of using multivariate methods to analyze the genomics of metabolic traits, at least in the context of locus discovery.

It should be noted that these multivariate methods and tests were developed by statistical geneticists to specifically increase the power of gene identification. In such "gene-centric" tests, the model that includes the effects of genotype on multiple traits is contrasted with the null model in which the gene has no effect on any trait analyzed. Although useful and powerful for genetic mapping, this approach may have limited interpretability in a context in which one is interested in the genetic control and biology of specific trait or a subset of traits (the "trait-centered" view). Several statistical methods have been suggested to address the question of which specific traits are affected in an analyzed ensemble (see for example [10,14]). One such method is based on conditional analysis [15], in which a "target trait" is analyzed as a genotype-dependent variable and related traits are included in the regression model as covariates. Such a modeling approach allows—at least in theory—one to rule out indirect genetic effects (e.g., effects that are in fact solely mediated through some other trait) and study only the genetic effects that directly affect the trait of interest.

Here, we present a statistical model in which a given trait depends on a genetic polymorphism and in which a number of related traits are included in the model as covariates. In this model, the relationship between the genotype and the trait of interest is our primary focus. Analyzing such a model allows us to identify the direct effect of genetics on the trait of interest. We first contrast our conditional genetic association (cGAS) approach with the standard model in which a trait of interest depends solely on genotype, without other traits used as covariates (i.e., the univariate genetic association—or uGAS—model). We do so by mathematically deriving expressions that allow us to examine the relative power of the uGAS and cGAS approaches, and we identify the situations in which these models are expected to yield different results.

As might be expected—and as demonstrated here—the choice of covariates plays a critical role in conditional analyses. First, we used the assumption that the covariates (i.e., biologically relevant traits) are known. Second, we explored the problem of selecting appropriate covariates, and we tested the approaches by performing a proof-of-principle study using metabolomics data consisting of 151 metabolites (Biocrates assay) obtained from the KORA F4 study (n=1785 individuals). Specifically, we selected covariates based on existing knowledge from metabolite

biochemical networks (BN-cGAS) and using a data-driven approach based on Gaussian Graphical Modeling (GGM-cGAS). Finally, we compare and discuss the obtained results, and we discuss possible applications for this analysis based on biologically and/or statistically relevant traits.

Results

The power of performing a conditional analysis of genetic associations

We start with the theoretical substantiation and identification of specific scenarios in which adjusting for biologically relevant covariates can modify the power of an association analysis.

Let us consider a trait of interest, *y*, covariate *c*, and genotype *g*. We can formulate this problem in terms of a linear regression as follows: $y = \beta_g * g + \beta_c * c + e$, where β_g and β_c are the effects of the genotype and covariate, respectively, and e is the residual noise. Without a loss of generality, we assume that all random variables in this equation are distributed with a mean of zero and a standard deviation of 1; if this is not the case, one would need to use partial regression coefficients and covariance instead of the correlation parameters that we use here. Given these assumptions made, the joint distribution of *y*, *g*, and *c* can be specified using a set of three correlation coefficients, ρ. Given specific parameters, the value of the "univariate" score test statistic for the association between *y* and *g* is calculated as follows: $T_u^2 = n\,\rho_{yg}^2/\sigma_u^2$, where *n* is the sample size and $\sigma_u^2 = 1 - \rho_{yg}^2$ is the residual variance of *y*. For the conditional test, $T_c^2 = n\,\beta_{yg}^2/\sigma_c^2 = n(\rho_{yg} - \beta_{yc}\rho_{cg})^2/\sigma_c^2$, where β represents the partial correlation coefficients from the conditional model and σ_c^2 is the residual variance of *y*. Consequently, the log-ratio of the conditional and univariate test statistics can be partitioned into two components:

$$\log\left(\frac{T_c^2}{T_u^2}\right) = \log\left(\frac{\sigma_u^2}{\sigma_c^2}\right) + \log\left(\left[1 - \frac{\beta_{yc}\rho_{cg}}{\rho_{yg}}\right]^2\right) \quad (1)$$

Because the first term in Eq. (1) is dependent only upon residual variances of the two models, we call this term the "noise" component. The second term depends upon the correlations between traits and between the traits and the genotype; we call this term the "pleiotropic" component. Because the noise component (σ_u^2/σ_c^2) is always $\geq$1, any possible decrease in the ratio between univariate and conditional tests is determined by the sign and the magnitude of the term $\beta_{yc}\rho_{cg}/\rho_{yg}$. If this term is negative, there will always be an increase in the power of the conditional analysis.

We can re-write $\beta_{yc}\rho_{cg}/\rho_{yg}$ as $\beta_{yc}\rho_{yc}^*$, where $\rho_{yc}^* = \rho_{cg}/\rho_{yg}$ is the quantity interpreted in a Mendelian randomization analysis as the effect of the covariate on the trait independent of non-genetic confounders [16]. Note that whereas ρ_{yc}^* reflects the covariance between the trait and the covariate (which is induced by the effect of the genotype), β_{yc} is related to the residual (in most cases, environmental) sources of covariance between *y* and *c*. Thus, we conclude somewhat surprisingly that when genotype-induced and environmental correlations are similar in sign (i.e., both are positive or both are negative), the product $\beta_{yc}\rho_{yc}^*$ is positive and the contribution of the

second term in Eq. (1) to the relative power is negative. Note that the contribution of the first term in Eq. (1) is always positive; therefore, even if $\beta_{yc}\rho^*_{yc}$ is positive, the power of a conditional analysis may still be higher than the power of a univariate analysis. In contrast, an "unexpected" product (in which the signs are different and hence $\beta_{yc}\rho^*_{yc}$ is negative) contributes positively to the relative power of the conditional model. Note that in such a situation, the power of a conditional analysis will always be higher than the power of a univariate analysis.

We can readily extend Eq. (1) to a situation in which *k* covariates are included in the conditional model. Denoting the coefficients of correlation between *g* and covariate *i* as ρ_{gi} and the partial coefficients of regression of *y* on covariate *i* as β_i yields the following equation:

$$\log\left(\frac{T_c^2}{T_u^2}\right) = \log\left(\frac{\sigma_u^2}{\sigma_c^2}\right) + \log\left(\left[1 - \frac{1}{\rho_{yg}}\sum_{i=1}^{k}\beta_i\rho_{gi}\right]^2\right) \quad (2)$$

When appropriate covariates are selected, performing cGAS using individual-level data becomes rather trivial and can be achieved using standard statistical and software tools in which one estimates the effects of a SNP and covariates. However, cGAS becomes somewhat less trivial if one chooses to use summary-level univariate GWAS data such as data available from previously published studies. The formalization of cGAS in terms of summary univariate GWAS statistics is described in **Supplementary Note 1.** Here, we used methods based on analyzing summary-level data.

Network-based selection of covariates

The ability to select appropriate covariates is extremely important, as it can have direct implications regarding the outcome of the analysis. If the biological/biochemical relationships between traits of interest are known and are summarized in a database(s), this knowledge can be used directly, for example by using all direct neighbors as covariates. We refer to this approach as a biochemical-network driven cGAS (BN-cGAS). Alternatively, the network can be reconstructed in a hypothesis-free, empirical manner from the data, for example using a Gaussian Graphical Model (GGM) [17]. We refer to this approach as a GGM-cGAS.

We compared cGAS and uGAS by performing a genome-wide analysis of genetic effects using summary-level data obtained from the KORA F4 study. This study included 151 metabolites measured in 1784 individuals using the Biocrates assay and imputed at 1,717,498 SNPs.

First, we examined the potential of using cGAS when the covariates are selected based on a known biochemical network (i.e., BN-cGAS). Thus, our analysis was restricted to a subset of 105 metabolites for which at least the one-reaction-step immediate biochemical neighbors are known [17]. This biochemical network incorporates only lipid metabolites, and the pathway

reactions cover two groups of pathways: (1) fatty acid biosynthesis reactions, which apply to the metabolite classes lyso-PC, diacyl-PC, acyl-alkyl-PC, and sphingomyelins; and (2) β-oxidation reactions that reflect fatty acid degradation and apply to acylcarnitines. The β-oxidation model consists of a linear chain of C2 degradation steps (C10 to C8 to C6, etc.). The number of covariates ranged from 1 to 4, with mean and median values of 2.48 covariates and 2 covariates, respectively.

Table 1 lists the 11 loci that were significant in either BN-cGAS or uGAS and fell into known associated regions (see **Supplementary Note 2**). Of these 11 loci, ten and nine loci could be identified by BN-cGAS and uGAS, respectively. Compared to uGAS, BN-cGAS identified one fewer locus (*ETFDH*), but identified two more (*ACSL1* for PC ae C42:5 and *PKD2L1* for lyso-PC a C16:1). It is interesting to note that for *ACSL1*, the effect of SNP rs4862429 on PC ae C42:5 was highly significant (p=7e-11) with BN-cGAS, but was not significant (p=0.7) with uGAS; this outcome is to be expected under the model of unexpected pleiotropy.

Next, to test whether using BN-cGAS increases the average power of the association analysis, we compared the BN-cGAS and uGAS chi-square test results for the loci listed in **Table 1**. Within a given locus, we compared the maximum test value. The ratio of the average maximum test statistic between BN-cGAS and uGAS was 1.59; however, a paired-sample Wilcoxon test comparing the best chi-square test results between BN-cGAS and uGAS was not significant (p=0.067).

For the SNPs listed in **Table 1**, we then used Eq. (2) to partition the log-ratio of the BN-cGAS and uGAS statistics values into "noise" and "pleiotropic" components. As shown in **Figure 1**, the ratio is determined primarily by the second (i.e., "pleiotropic") term in Eq. (2). Moreover, with the exception of the *SLC22A4* locus, the SNP-trait pairs for which BN-cGAS had increased power are the pairs in which the second term in Eq. (2) is either positive or close to zero. In contrast, in the SNP-trait pairs that were not identified using BN-cGAS, the "pleiotropic" term in Eq. (2) had a strong negative contribution.

Next, we investigated the variance-covariance structure of the loci with positive and negative pleiotropic terms. We therefore selected a locus in which the pleiotropic component's contribution to power was positive (rs174547 at *FADS1*) and a locus in which the pleiotropic component's contribution to power was negative (rs8396 at *ETFDH*). **Figure 2** shows the corresponding correlations between the SNP, the trait, and the covariates involved, together with the partial coefficients for the conditional regression of the trait on the SNP and the covariates. With respect to *FADS1* (**Figure 2A**), the correlations between the SNP and the trait (lyso-PC a C20:4) and between the SNP and the covariate (lyso-PC a C20:3) are in opposite directions, generating negative genetically induced covariance between lyso-PC a C20:4 and lyso-PC a C20:3. In contrast, the residual correlation between the trait and the covariate is positive.

Therefore, the value of the partial coefficient of regression between the SNP and lyso-PC a C20:4, conditional on lyso-PC a C20:3, is greater than that of the coefficient of regression without covariates.

With respect to the second example, *ETFDH* (**Figure 2B**), we found that the conditional regression of C10 on rs8396 and two covariates (C8 and C12, two medium-chain acylcarnitines) led to a smaller SNP partial regression coefficient compared to an unconditional regression; this is because all of the terms in $\sum_{i=1}^{k} \beta_i \rho_{gi} / \rho_{yg}$ are positive.

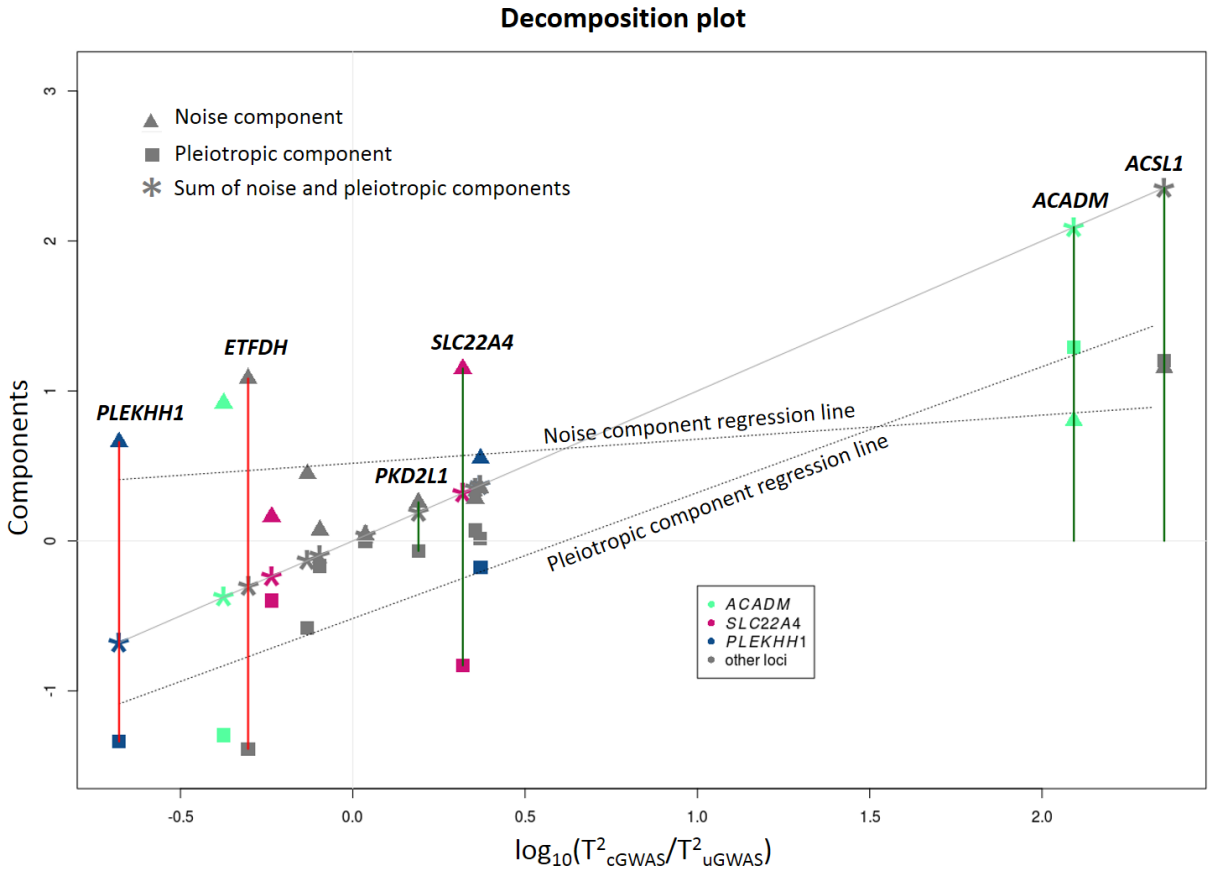

Figure 1. Decomposition of the log-T^2 ratio for cGAS and uGAS into pleiotropic and noise components. Vertically grouped trios (each composed of a square, triangle, and asterisk) correspond to one of fourteen associations in Table 1. The position of a trio on the *x*-axis corresponds to the log-ratio between conditional and univariate test statistic. On the *y*-axis, the asterisk corresponds to the log-ratio. The value of the pleiotropic component is depicted by a square, and the value of the noise component is depicted by a triangle. Each trio is shown in gray, except the trios representing the *ACADM*, *SLC22A4*, and *PLEKHH1* loci, for which we have two different associations. The two dotted lines correspond to the regression lines for the two components. The three dark-green vertical lines indicate the associations that were significant in the cGAS analysis but not in the uGAS analysis, and the two dark-red lines indicates the associations that were significant only in the uGAS analysis.

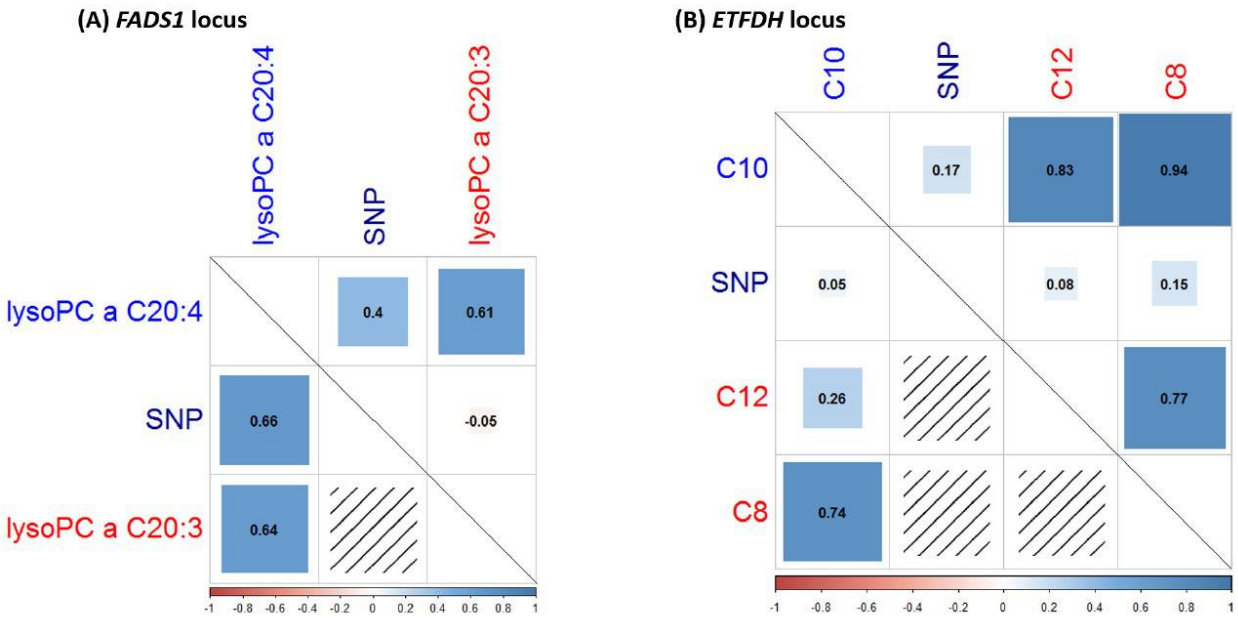

Figure 2. Matrix of correlations (above diagonal line) and the partial coefficients of regression of the trait of interest on the SNP genotype and covariate(s) (the first column below diagonal line) for the *FADS1* (A) and *ETFDH* (B) loci. Names of traits used as covariates are in red. The number in a cell indicates the value of correlation (partial regression coefficient). The area of a square is proportional to the absolute value of correlation (partial regression coefficient); the effect magnitude is also reflected by square's color (the scale provided at the bottom of the graph). The *FADS1* locus represents scenario in which the pleiotropic term in Eq. (2) is strongly positive, while for *ETFDH* this term is negative.

Although using a known biochemical network to select covariates has many advantages, it may be somewhat unpractical and perhaps even harmful, as our biochemical knowledge is still relatively incomplete. Therefore, we explored the potential of performing a cGAS in which the covariates are selected using a data-driven approach (GGM-cGAS). The network of metabolites was reconstructed using Gaussian Graphical Models based on partial correlations. For a given metabolite, we selected covariates based on significant partial correlations. Specifically, we used the following threshold as proposed previously [17]: a *p*-value $\leq$ (0.01/number of calculated partial correlations), which corresponds to a cut-off at $p \leq 8.83 \times 10^{-7}$. The network used in our analysis is shown in **Supplementary Figure S1**.

To compare GGM-cGAS with BN-cGAS, we used the same set of metabolites that we used for BN-cGAS to run our GGM-cGAS analysis; these results are presented in **Supplementary Table S1**. We found 16 SNP-trait pairs clustered at 10 loci that were detected by either GGM-cGAS or BN-cGAS. More covariates were included in the GGM-cGAS analysis (ranging from 1

to 18, with mean and median values of 7.6 covariates and 7 covariates, respectively) than in the BN-cGAS analysis. Thus, we predicted that GGM-cGAS would have relatively more power than BN-cGAS due to reduced noise (term 1 in Eq. (2)); on the other hand, GGM-cGAS might lose power because of reduced occurrence of unexpected pleiotropy (term 2 in Eq. (2)).

For the best SNP-trait pairs detected by GGM-cGAS or BN-cGAS, we computed the components in Eq. (2) and compared these components using a paired-sample Wilcoxon test. We found that the noise component in Eq. (2) was always larger for GGM-cGAS, with a mean difference of 0.66 ($p=3 \times 10^{-5}$). Moreover, the second "pleiotropic" component in Eq. (2) was on generally smaller in GGM-cGAS than for BN-cGAS, with a mean difference of -0.54 ($p=0.013$); nevertheless, for three out of 16 GGM-cGAS SNP-trait pairs, the pleiotropic component was positive. The average chi-square value was 33% smaller for GGM-cGAS than for BN-cGAS, indicating an average loss of power (although this loss was not significant; $p=0.5$ based on a paired Wilcoxon test), yet 22% larger for GGM-cGAS than for uGAS ($p=0.8$ based on a paired Wilcoxon test). We therefore conclude that although GGM-cGAS may not serve as an ideal proxy for analyzing a bona fide biochemical network, it can still have increased power due to reduced target trait residual variance and the potential to detect unexpected pleiotropy.

Next, we investigated further the potential of using cGAS under realistic conditions to a full extent by analyzing all 151 available metabolites using GGM-cGAS and comparing these results with the results of uGAS (**Table 2** and **Supplementary Figure S2)**. In total, uGAS detected 15 loci at the genome-wide significance level $p \leq 5 \times 10^{-8}/151$ (i.e., $p<3.3 \times 10^{-10}$). On the other hand, GGM-cGAS identified 19 significant loci using the same threshold. As expected, the standard errors of the genetic effect estimates were smaller for GGM-cGAS than for uGAS (**Table 2** and **Supplementary Figure S3**). A total of 14 loci were detected by both uGAS and GGM-cGAS. GGM-cGAS failed to identify one locus that was identified by uGAS (C5:1-DC at rs2943644), but identified five loci that were missed by uGAS. Three of the five loci identified solely by GGM-cGAS affect amino acids, and the remaining two loci affect acylcarnitines. It is important to note that the loci identified by BN-cGAS (when we analyzed 105 metabolites) are a subset of the 19 loci that were identified by GGM-cGAS (when we used all 151 metabolites).

Finally, we searched the available literature for the loci listed in **Table 2** (see **Supplementary Note 2** for details). From the 20 loci that we report here, 15 were found to be significant at the genome-wide level in a recent large (n=7478) meta-analysis of Biocrates metabolomics data reported by Draisma et al. [7]. Some of the metabolites analyzed in our study were not analyzed by Draisma et al. [7]; nevertheless, for 11 out of these 15 loci, we observed a significant association for the same SNP-metabolite pair; for three loci, the strongest association was with a metabolite in the same class, and for one locus the strongest association was with a

metabolite from a different lipid class (see **Supplementary Table S2**). For the other five loci that were not significant in the study by Draisma et al. [7], we determined whether these five loci were significant and replicated in a study by Tsepilov et al. [18]. It should be noted that Tsepilov et al. analyzed the ratios of metabolites and also used the KORA F4 data set in their discovery stage, although they used another cohort (TwinsUK) for replication. Of these five loci, two were also significant in the study by Tsepilov et al. [18]; moreover, for both of these loci the metabolite analyzed in our study was included in the ratios analyzed by Tsepilov et al. One of the five loci was associated with the same trait in two other studies [19,20]. Finally, we found no prior published evidence of any association with metabolites for rs2943644 (*LOC646736*) or rs17112944 (*LOC728755*); therefore, we conclude the observed associations with rs17112944 and rs2943644 as likely false positives, and these two loci were excluded from further consideration.

Table 1. Eleven loci identified by BN-cGAS and uGAS on metabolites for which at least one one-reaction-step neighbor was available.

							uGAS		cGAS		
Locus	**SNP**	**Metabolite**	**chr:pos**	**Gene**	**effA/refA**	**EAF**	**beta (se)**	**P-value**	**Beta (se)**	***P*-value**	**N_{cov}**
uGAS & cGAS											
1	rs211718	C8	1:75879263	*ACADM*	T/C	0.30	-0.45 (0.034)	3.26E-37	-0.10 (0.012)	4.83E-17	1
1	rs211718	C12	1:75879263	*ACADM*	T/C	0.30	-0.04 (0.036)	2.19E-01	0.20 (0.014)	1.67E-40	3
2	rs7705189	PC ae C42:5	5:131651257	*SLC22A4*	G/A	0.47	0.15 (0.034)	8.65E-06	0.06 (0.009)	1.49E-10	3
2	rs419291	C5	5:131661254	*SLC22A4*	T/C	0.38	0.26 (0.035)	7.03E-14	0.17 (0.029)	1.01E-08	1
3	rs9368564	PC aa C42:5	6:11168269	*ELOVL2*	G/A	0.25	-0.29 (0.039)	1.14E-13	-0.15 (0.024)	1.63E-10	3
4	rs12356193	C0	10:61083359	*SLC16A9*	G/A	0.17	-0.51 (0.046)	1.84E-27	-0.42 (0.042)	1.67E-22	1
5	rs174547	lyso-PC a C20:4	11:61327359	*FADS1*	C/T	0.70	0.61 (0.033)	1.24E-69	0.66 (0.024)	2.96E-141	1
6	rs2066938	C4	12:119644998	*ACADS*	G/A	0.27	0.73 (0.033)	2.42E-93	0.72 (0.032)	2.13E-100	1
7	rs10873201	PC ae C36:5	14:67036352	*PLEKHH1*	T/C	0.45	-0.26 (0.034)	4.37E-14	-0.21 (0.018)	2.38E-30	2
7	rs1077989	PC ae C32:2	14:67045575	*PLEKHH1*	C/A	0.46	-0.30 (0.034)	2.23E-18	-0.06 (0.016)	5.33E-05	3
8	rs4814176	PC ae C40:2	20:12907398	*SPTLC3*	T/C	0.36	0.24 (0.035)	5.74E-12	0.25 (0.023)	1.58E-25	4
Only uGAS											
9	rs8396	C10	4:159850267	*ETFDH*	C/T	0.71	0.26(0.037)	2.11E-12	0.05 (0.011)	6.67E-07	2
Only cGAS											
10	rs4862429	PC ae C42:5	4:186006834	*ACSL1*	T/C	0.31	0.02(0.037)	6.62E-01	-0.06 (0.010)	6.57E-11	3
11	rs603424	Lyso-PC a C16:1	10:102065469	*PKD2L1*	A/G	0.80	0.23(0.042)	5.34E-08	0.21 (0.031)	1.39E-11	1

Notes: The best SNP-metabolite pair is shown for each locus. chr:pos refers to the physical position of the SNP; EAF, effect allele frequency; beta (se), the estimated effect and standard error of the SNP; effA/refA, effect allele/reference allele; *P*-value, the *p*-value of the additive model; Gene, the most likely (according to DEPICT) associated gene in the region; N_{cov}, the number of covariates used in cGAS.

Table 2. Twenty loci identified by GGM-cGAS and uGAS.

							uGAS		cGAS		
Locus	**SNP**	**Metabolite**	**chr:pos**	**Gene**	**effA/refA**	**EAF**	**beta (se)**	**P-value**	**beta (se)**	**P-value**	**N_{cov}**
uGAS & cGAS											
1	rs211718	C6 (C4:1-DC)	1:75,879,263	*ACADM*	T/C	0.30	-0.48 (0.034)	4.64E-42	-0.13 (0.017)	2.00E-13	7
1	rs7552404	C6 (C4:1-DC)	1:75,908,534	*ACADM*	G/A	0.30	-0.48 (0.034)	3.10E-42	-0.12 (0.017)	3.25E-13	7
2	rs483180	Ser	1:120,069,028	*PHGDH*	G/C	0.30	-0.24 (0.037)	3.34E-11	-0.24 (0.028)	1.50E-17	2
2	rs477992	Ser	1:120,059,099	*PHGDH*	A/G	0.70	0.24 (0.037)	5.15E-11	0.24 (0.028)	5.82E-18	2
3	rs2286963	C9	2:210,768,295	*ACADL*	G/T	0.63	-0.49 (0.032)	1.10E-49	-0.48 (0.027)	1.48E-67	3
4	rs8396	C10	4:159,850,267	*ETFDH*	C/T	0.71	0.26 (0.037)	2.02E-12	0.04 (0.010)	1.49E-05	8
4	rs8396	C7-DC	4:159,850,267	*ETFDH*	C/T	0.71	-0.09 (0.037)	1.67E-02	-0.13 (0.020)	3.29E-11	8
5	rs419291	C5	5:131,661,254	*SLC22A4*	T/C	0.38	0.26 (0.035)	7.03E-14	0.17 (0.026)	2.28E-10	3
5	rs270613	C5	5:131,668,482	*SLC22A4*	A/G	0.61	-0.26 (0.035)	7.93E-14	-0.17 (0.026)	8.48E-11	3
6	rs9393903	PC aa C42:5	6:11,150,895	*ELOVL2*	A/G	0.75	0.29 (0.039)	2.19E-13	0.18 (0.020)	4.51E-19	6
6	rs9368564	PC aa C42:5	6:11,168,269	*ELOVL2*	G/A	0.25	-0.29 (0.039)	1.14E-13	-0.19 (0.021)	7.84E-19	6
7	rs816411	Ser	7:56,138,983	*PHKG1*	C/T	0.51	-0.22 (0.034)	2.15E-10	-0.19 (0.026)	5.16E-13	2
7	rs1894832	Ser	7:56,144,740	*PHKG1*	C/T	0.51	0.21 (0.034)	3.23E-10	0.19 (0.026)	1.69E-13	2
8	rs12356193	C0	10:61,083,359	*SLC16A9*	G/A	0.17	-0.51 (0.046)	1.84E-27	-0.27 (0.034)	9.72E-16	3
9	rs174547	lyso-PC a C20:4	11:61,327,359	*FADS1*	C/T	0.70	0.61 (0.033)	1.44E-69	0.07 (0.011)	1.41E-10	9
9	rs174556	PC ae C44:4	11:61,337,211	*FADS1*	T/C	0.27	0.09 (0.038)	1.55E-02	0.21 (0.014)	3.16E-46	3
10	rs2066938	C4	12:119,644,998	*ACADS*	G/A	0.27	0.73 (0.033)	5.87E-94	0.71 (0.025)	1.31E-151	2
11	rs12879147	PC aa C28:1	14:63,297,349	*SYNE2*	A/G	0.85	-0.46 (0.050)	2.07E-19	-0.12 (0.019)	5.94E-11	14
11	rs17101394	SM(OH) C14:1	14:63,302,139	*SYNE2*	A/G	0.83	-0.32 (0.050)	1.00E-10	-0.10 (0.011)	1.17E-17	7
12	rs1077989	PC ae C36:5	14:67,045,575	*PLEKHH1*	C/A	0.46	-0.26 (0.034)	3.42E-14	-0.08 (0.010)	8.25E-16	10
12	rs1077989	PC ae C32.2	14:67,045,575	*PLEKHH1*	C/A	0.46	-0.30 (0.034)	2.23E-18	-0.05 (0.016)	1.31E-03	6
13	rs4814176	SM(OH).C22:1	20:12,907,398	*SPTLC3*	T/C	0.36	0.03 (0.035)	4.51E-01	-0.07 (0.009)	1.10E-16	10

13	rs4814176	SM(OH) C24:1	20:12,907,398	*SPTLC3*	T/C	0.36	0.24 (0.035)	5.40E-12	0.09 (0.013)	3.04E-11	9
14	rs5746636	Pro	22:17,276,301	*PRODH*	T/G	0.24	-0.31 (0.039)	3.00E-15	-0.32 (0.034)	1.91E-20	2
Only uGAS											
15	rs2943644	C5:1-DC	2:226,754,586	*LOC646736*	C/T	0.68	0.32 (0.042)	5.14E-14	0.09 (0.022)	3.58E-05	5
Only cGAS											
16	rs1374804	Gly	3:127,391,188	*ALDH1L1*	A/G	0.64	0.20 (0.036)	1.88E-08	0.21 (0.030)	8.08E-13	3
17	rs4862429	PC ae C42:5	4:186,006,834	*ACSL1*	T/C	0.31	0.02 (0.037)	6.62E-01	-0.06 (0.008)	1.25E-12	8
18	rs603424	C16:1	10:102,065,469	*PKD2L1*	A/G	0.80	0.16 (0.042)	9.51E-05	0.14 (0.018)	1.32E-13	9
19	rs2657879	Gln	12:55,151,605	*GLS2*	G/A	0.21	-0.24 (0.042)	2.82E-08	-0.27 (0.031)	9.37E-18	5
20	rs17112944	C6:1	14:27,179,297	*LOC728755*	A/G	0.90	-0.28 (0.059)	2.09E-06	-0.21 (0.032)	1.38E-10	9

Notes: The best SNP-metabolite pair is shown for each locus. chr:pos refers to the physical position of the SNP; EAF, effect allele frequency; beta (se), the estimated effect and standard error of the SNP; effA/refA, effect allele/reference allele; *P*-value, the *p*-value of the additive model; Gene, the most likely (according to DEPICT) associated gene in the region; N_{cov}, the number of covariates used in cGAS.

Discussion

We report a new "trait-centric" approach for analyzing genetic determinants of multivariate "omics" traits by performing a network-based conditional genetic association analysis (cGAS). In the context of metabolomics, for each trait we selected a set of other metabolites to be used as covariates in our genetic association analysis. The selection of covariates can be either mechanistic (e.g., based on known biological relationships between traits of interest) or data-driven (e.g., based on partial correlations). Importantly, this approach can use either individual-level or summary-level data. We first mathematically compared the power of conditional and standard single-trait genetic association analyses (univariate genetic association, uGAS), and we identified scenarios in which these analyses are expected to produce different results; next, we applied cGAS to 151 metabolomics traits (Biocrates panel) in a large (n=1784 individuals) population-based KORA cohort.

We found that the log-ratio between the cGAS and uGAS test statistic can be decomposed in a "noise" component (which depends on residual variance of the trait and is always positive) and a "pleiotropic" component. The pleiotropic component is negative in cases in which genetically induced covariance (between the trait of interest and the trait used as the covariate) and the residual covariance have the same sign (i.e., act in the same direction). The pleiotropic component is positive in cases in which the genetically induced covariance and residual covariance act in opposite directions.

Should one expect that genetically induced and residual covariance act in the same or opposite directions? In the context of complex polygenic traits, one would expect that genetic and environmental correlations have the same sign. This is reflected in animal breeding studies; for a recent example in humans, see [21]. It should be noted that a negative sign for the pleiotropic component does not necessarily indicate higher power of the uGAS, as the noise component may still dominate the relative non-centrality parameter. This will happen, for example, when ρ_{cg} (the effect of the genotype on the covariate) is small while β_{yc} (partial residual regression between the trait and covariate) is relatively large, thereby reducing σ_c^2.

Nevertheless, in the case of metabolomic traits, genetic and environmental sources do not necessarily generate consistent covariance. Moreover, for a given locus that affects the activity of an enzyme involved in a biochemical reaction, the unexpected inconsistency between genetically induced covariance and residual covariance may not be so unexpected after all. Indeed, consider an allele associated with an increased activity of an enzyme that converts substrate A into product B. One would expect that the levels of A and B are positively correlated; one would also expect

that the allele is positively correlated with the level of product B and negatively correlated with the level of substrate A. This is precisely the scenario that yields a positive value for the second term in Eq. (1), thus providing an additional increase in power above and beyond the power provided by the first term in Eq. (1) (noise reduction).

Our empirical investigation of real data on the genetic association between the genome and metabolites confirmed the existence of both scenarios. An extreme example of concordance between genetic covariance and residual covariance is provided by the effects of rs8396 on C10, with C8 and C12 used as covariates (see Figure 2B). The *ETFDH* gene, which was prioritized by DEPICT as the best candidate in this region (with a false-discover rate <5%), encodes the enzyme electron transfer flavoprotein (ETF) dehydrogenase, which plays a role in mitochondrial fatty acid oxidation. During this process, the acyl group is transferred from a long chain acylcarnitine to a long-chain acetyl-CoA, which is then catabolized. ETF dehydrogenase participates in the catabolic process by transferring electrons from acyl-CoA dehydrogenase to the oxidative phosphorylation pathway. Thus, the *ETFDH* gene should affect all forms of long-chain acylcarnitines in the same way, and we can expect that the pleotropic effect of this gene on the acylcarnitines in our example (C8, C10, C12, etc.) will be unidirectional. The presence of unidirectional genetic effects and the positive correlation between these acylcarnitines makes the second term in Eq. (2) negative, which determines that—in this situation—univariate GAS has more power than cGAS.

An empirical example of discordance between genetically induced covariance and residual covariance is provided by the effects of the SNP rs174547 on lyso-PC a C20:4, with lyso-PC a C20:3 used as a covariate. This SNP exhibits opposite correlations with lyso-PC a C20:4 and lyso-PC a C20:3, resulting in negative genetically induced covariance between these traits. At the same time, the residual correlation between these traits is positive, resulting in steep increase in the power of conditional analysis. In this region, the *FADS1/2/3* gene cluster is an attractive candidate, providing the detected model with biological relevance. The *FADS1* gene encodes the enzyme fatty acid desaturase 1, whereas the two traits differ by only one double bond. Thus, this example mimics perfectly the biochemical scenario in which we would expect a conditional analysis to have increased power.

The trait-centric methods considered here provide an attractive framework to identify and study direct genetic effects on a trait of interest. Conditional analysis is an attractive option in cases in which we wish to clearly interpret the results in terms of the effect of the genotype on a particular trait. Such specific interpretation may be important when comparing genetic association results obtained for our trait of interest with results obtained for other traits (e.g., using the methods described in [22–24]). It should be noted, though, that a trait-centric approach is not intended to maximize the power of identifying genes that affect metabolomics as a whole. Such a gene-centric

view would favor analysis using joint—and not conditional—modeling of sets of traits. Such an approach can maintain power across a wide range of scenarios, including the scenario of concordance between genetically induced and residual covariance [13]. In this gene-centric framework, other formulations of conditional analysis have also been proposed [25] in order to specifically increase power of gene identification by selecting covariates that—using our terminology—affect the "noise reduction" component of the model while avoiding the problems associated with the pleiotropic component.

The proper selection of sets of biologically related traits is extremely important for the conditional genetic association analysis method described here, as well as for multivariate methods that model the joint effects of genotype on an ensemble of traits. Here, we considered two alternative approaches—knowledge based and data-driven—to finding the networks of related traits, with a subnetwork centered around a trait of interest used as the analyzed set. In principle, in the context of analyzed metabolomics data, the knowledge-based network approach has slightly higher power in the context of trait-centric genetic association analysis. However, we believe that our analysis revealed that both approaches are suboptimal. The knowledge-based network reconstruction has many advantages, but it may be somewhat unpractical, as our biochemical knowledge is still relatively incomplete. Secondly, by reconstructing the network while relying only on current knowledge, we may be missing new knowledge that may be revealed by the data. Finally, by including neighbors that are based only on biochemical information, we may miss covariance induced by technical confounders; adjusting for this may increase the power of analysis [25]. Learning the network from the same data that were used for genetic analysis has the disadvantages of potentially ignoring existing knowledge and being sensitive to sample size. Finally, we note that the total observed correlation between metabolites is determined by the balance between genetic and environmental sources of covariance; it is possible to imagine a situation in which total correlation is smaller than one or more of its components, and our analysis provides examples of such a situation. We may speculate that—ideally—one should use a method that allows one to combine prior knowledge and new information obtained from the data, thereby allowing the simultaneous learning of the structure of dependencies between different metabolites and between the metabolites and the genome. Such learning from the data while allowing for the incorporation of previous knowledge (e.g., biochemical relations between traits) might be achieved by applying, e.g. a machine-learning approach that allows for differential shrinkage. It is also important to note that the proper application of such an approach would require the availability of vast samples of data, thereby allowing for separate training, validation, verification, testing, and replication of detected dependencies and associations.

Materials and Methods

KORA study

The KORA study (Cooperative Health Research in the region of Augsburg) is a series of population-based studies in the region of Augsburg in Southern Germany [26]. KORA F4 is a follow-up survey (conducted from 2006 through 2008) of the baseline KORA S4 survey, which was conducted from 1999 through 2001. All study protocols were approved by the ethics committee of the Bavarian Medical Chamber, and all participants provided written informed consent.

The concentration of 163 metabolites were measured in 3061 serum samples obtained from KORA F4 participants using flow injection electrospray ionization tandem mass spectrometry and the AbsoluteIDQ p150 Kit (Biocrates Life Sciences AG, Innsbruck, Austria) [27]. After applying quality control screening, a total of 151 metabolite measurements were used in our analysis. Details regarding the methods and quality control of the metabolite measurements, as well as details regarding the metabolite nomenclature, have been published previously [27]. The nomenclature for the metabolites in this study is provided in **Supplementary Table S3**.

Genotyping was performed using the Affymetrix 6.0 SNP array (534,174 SNP markers after quality control), with further imputation using HapMap2 (release 22) as a reference panel, resulting in a total of 1,717,498 SNPs (for details, see ref. [28]). Both the metabolite concentrations and genotype were available for 1785 participants in the KORA F4 study.

Statistical analysis

Partial correlation coefficients and their *p*-values were calculated using the "ppcor" package [29] in R. Graphical representations were generated using the "ggm" [30] package in R. Consistent with previous studies [17], we considered a partial correlation coefficient to be significant at $p < 0.01/(151*150/2)$ (i.e., $p<8.83 \times 10^{-7}$).

For the GWAS analysis, we used OmicABEL software [31]. Prior to GWAS, all traits were first adjusted for the participant's sex, age, and batch effect; subsequently, the residual traits were transformed using an inverse-normal transformation [32]. The genotypes from the KORA F4 cohort were used. Only SNPs that had a call rate ≥0.95, $R^2 \geq 0.3$, Hardy–Weinberg equilibrium (HWE) $p \geq 10^{-6}$, and MAF ≥0.1 (1,717,498 SNPs in total) were included in the analysis. The genomic control method was used to correct for any possible inflation of the test statistics. The genomic control [33] lambda value for all traits was between 1.00 and 1.03.

In a specific analysis (i.e., cGAS or uGAS), we defined independent loci as groups of genome-wide significant associations that were separated by at least 500 kb or were located on

different chromosomes. The strongest association (i.e., the association with the lowest *p*-value) was selected to represent this locus. The cGAS and uGAS results were considered to reflect different loci if the strongest associations were in loci that were separated by at least 500. The threshold for the genome-wide significance for 151 traits was set to $p=5 \times 10^{-8}/151$ (i.e., $p=3.31 \times 10^{-10}$).

When partitioning the log(cGAS/uGAS) test statistics into the noise and pleiotropic components (see Eq. (2) and **Figure 1**), we used all known loci that were significant in either the cGAS or uGAS analysis (see Table 1). If a locus included two SNPs associated with different traits, we included both associations during partitioning. If a locus included two SNPs associated with the same trait, to be conservative we included only the locus with the lower uGAS *p*-value during partitioning. After partitioning, we compared the pleiotropic and noise components using the paired-samples Wilcoxon test. For comparing the chi-square test results for the two methods, for each locus we first selected the method that yielded the strongest association (and hence the largest chi-square value). We compared that chi-square value with the maximal chi-square value observed for the second method within a 500-kb region centered around the strongest association observed using the first method.

In silico functional annotation

We conducted functional annotation for our findings. To prioritize genes in associated regions, gene set enrichment, and tissue/cell-type enrichment analyses, we used DEPICT software [34] (release 140721) with the following settings: flag_loci = 1; flag_genes = 1; flag_genesets = 1; flag_tissues = 1; param_ncores = 2; and further manual annotation (h37 assembly). All 27 SNPs (clustered in 20 loci) identified by cGAS or uGAS (see **Table 2**) were included in the analysis. If more than one gene was annotated for a SNP by DEPICT, we selected the gene with the lowest nominal DEPICT P-value. In most cases, the results of manual annotation matched the annotation results using DEPICT annotation (see **Supplementary Note 2**). In addition, we looked up each SNP using the Phenoscanner [35] database to check whether it was previously reported to be associated with metabolic traits at $p<5 \times 10^{-8}$ and proxy $r^2 <0.7$.

Additional Files

Supplementary Note 1 – cGAS using summary level data

Supplementary Note 2 – Literature search for loci identified by cGAS and uGAS

Supplementary Tables

Supplementary Table S1 – BN- cGAS and GGM- cGAS for 105 metabolites

Supplementary Table S2 – GGM-cGAS and uGAS for 151 metabolites

Supplementary Table S3 - List of metabolites measured using the AbsoluteIDQ p150 Kit

Supplementary Figures

Supplementary Figure S1 – Partial correlations network

Supplementary Figure S2 – Manhattan plots for cGAS and uGAS for 151 metabolites

Supplementary Figure S3 – Comparison of effect estimates and their standard errors for SNPs from Table 2

Abbreviations

GWAS – genome-wide association study

cGAS – conditional GWAS

uGAS – univariate GWAS (trait-by-trait)

BN-cGAS – cGAS based on biochemical networks

GGM-cGAS – Gaussian Graphical Modeling cGAS based on partial correlations network

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Authors Contributions

YT, CG, and YA designed and supervised the study; PC, CP, JA, KG, and RW-S collected the data; CG and KS contributed data for the analysis; YT, OZ, and SS analyzed the data; YT, YA, CG, OZ, JK, and KS discussed and interpreted the results; YT, OZ, CG, and YA wrote the manuscript. All authors contributed to and approve the final version of the manuscript.

Availability of Data and Materials

The code produced in relation to this work is distributed under the LGPL license and is available from [WHILE MANUSCRIPT IS UNDER REVIEW, THE LINK TO THE DATA IS ONLY AVAILABLE TO THE REVIEWERS FROM THE EDITOR]. All summary statistics and association data that are necessary to reproduce our results are licensed under CC0 and are accessible online at [WHILE MANUSCRIPT IS UNDER REVIEW, THE LINK TO THE DATA IS ONLY AVAILABLE TO THE REVIEWERS FROM THE EDITOR]. The informed consent given by the KORA study participants does not cover the posting of participant-level phenotype or genotype data in public databases. However, the KORA data are available upon request from KORA-gen (https://www.helmholtz-muenchen.de/en/kora/index.html). Requests can be submitted online and are subject to approval by the KORA board.

Competing Interests

Y. Aulchenko is the founder and co-owner of PolyOmica, a private research organization that specializes in computational and statistical (gen)omics.

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Supplementary Table 1

Supplementary Table 2

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