Identification of seven novel loci associated with amino acid levels using single-variant and gene-based tests in 8545 Finnish men from the METSIM study

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Abstract

Comprehensive metabolite profiling captures many highly heritable traits, including amino acid levels, which are potentially sensitive biomarkers for disease pathogenesis. To better understand the contribution of genetic variation to amino acid levels, we performed single variant and gene-based tests of association between nine serum amino acids (alanine, glutamine, glycine, histidine, isoleucine, leucine, phenylalanine, tyrosine, and valine) and 16.6 million genotyped and imputed variants in 8545 non-diabetic Finnish men from the METabolic Syndrome In Men (METSIM) study with replication in Northern Finland Birth Cohort (NFBC1966). We identified five novel loci associated with amino acid levels ($P = < 5\times10^{-8}$): *LOC157273/PPP1R3B* with glycine (rs9987289, $P = 2.3\times10^{-26}$); *ZFHX3* (chr16:73326579, minor allele frequency (MAF) = 0.42%, $P = 3.6\times10^{-9}$), *LIPC* (rs10468017, $P = 1.5\times10^{-8}$), and *WWOX* (rs9937914, $P = 3.8\times10^{-8}$) with alanine; and *TRIB1* with tyrosine (rs28601761, $P = 8\times10^{-9}$). Gene-based tests identified two novel genes harboring missense variants of MAF <1% that show aggregate association with amino acid levels: *PYCR1* with glycine ($P_{gene} = 1.5\times10^{-6}$) and *BCAT2* with valine ($P_{gene} = 7.4\times10^{-7}$); neither gene was implicated by single variant association tests. These findings are among the first applications of gene-based tests to identify new loci for amino acid levels. In addition to the seven novel gene associations, we identified five independent signals at established amino acid loci, including two rare variant signals at GLDC (rs138640017, MAF=0.95%, $P_{conditional} = 5.8\times10^{-40}$) with glycine levels and *HAL* (rs141635447, MAF = 0.46%, $P_{conditional} = 9.4\times10^{-11}$) with histidine levels. Examination of all single variant association results in our data revealed a strong inverse relationship between effect size and MAF ($P_{trend}<0.001$). These novel signals provide further insight into the molecular mechanisms of amino acid metabolism and potentially, their perturbations in disease.

Introduction

Amino acid levels are highly heritable biomarkers of human disease (1) that have been implicated in a range of clinical syndromes including type 2 diabetes/insulin resistance (2–4), liver disease (5) and Alzheimer's disease (6). Previous studies have together identified >200 common variant signals associated with amino acid levels (7–20). However, the contribution of genetic variation to amino acid level trait variance, and the role of rare genetic variation in particular, is not fully understood.

One method of assessing rare variant associations is through aggregation of multiple rare variants into a single test (21). One such approach groups rare, protein-altering variants into one test for association for each gene (21). This method has been used successfully to identify gene-based associations with *HAL* for histidine levels and with *PAH* for phenylalanine levels (17). Notably, this result occurred in the absence of any single variant reaching genome-wide significance in either *HAL* or *PAH*, highlighting the importance of gene-based tests in identifying novel genetic loci for complex traits.

In this study, we performed genome-wide single-variant and gene-based association analysis in 8545 non-diabetic Finnish men from the METabolic Syndrome In Men (METSIM) study to identify genetic associations with serum amino acid levels. We identified seven novel amino acid loci—five from single-variant tests [of which two signals replicated in the Northern Finnish Birth Cohort 1966 (NFBC1966) dataset] and two from gene-based associations. We also performed analyses conditioned on all previously known amino acid genome-wide association studies (GWAS) signals and identified five additional novel and independent signals in known amino acid loci, of which three replicated in the NFBC1966 data. In total, we identified five novel and replicated loci-amino acid associations, and two novel gene-based associations. These results help clarify the role of the specific variants and genes in amino acid homeostasis.

Results

GWAS for nine amino acids levels

To identify genetic variants associated with the nine amino acid traits measured in the METSIM study (alanine, glutamine, glycine, histidine, isoleucine, leucine, phenylalanine, tyrosine and valine; see Supplementary Material, Figs S1–S3 and Supplementary Material, Table S1), we analyzed 16.6M genotyped and imputed variants in 8545 non-diabetic Finnish men of mean age 57 years and mean BMI 27 kg/m^2 (see Supplementary Material, Table S1).

We identified 2428 unique variants associated with at least one amino acid trait ($P < 5\times10^{-8}$), and a total of 2580 variant-trait associations (see Supplementary Material, Table S2 and Figs S4 and S5). Of the 2580 variant-trait associations, the majority were with glycine (1403 variants), followed by tyrosine (560), glutamine (164), alanine (95), leucine (89), isoleucine (87), phenylalanine (67), valine (62) and histidine (53). We present a summary of the variants and their distributions in independent loci in Supplementary Material, Table S3.

We estimated for each amino acid trait the phenotypic variation that genetic variants explained from 10.4% (histidine) to 28.5% (glycine) of variation (see Supplementary Material, Table S4).

Restricting analysis to genome-wide significant variant-trait associations ($P < 5 \times 10^{-8}$, see Supplementary Material, Table S2), the proportion of phenotypic variation explained by significantly associated variants ranged from 1.3% (leucine) to 18.3% (glycine) (see Supplementary Material, Table S4).

We attempted to validate genotypes at three rare imputed trait-associated variants with MAF < 0.5% (see Supplementary Material, Table S5). We confirmed two variants with no discordance between imputed and sequenced genotypes: rs141635447 (0/74 discordant) and chr16: 73326579 (0/67). Variant chr3: 125173967 showed a discordance rate of 39% (24/61), and was thus removed from subsequent analyses.

Single-variant analysis identifies novel associations at *LOC157273/PPP1R3B*, *WWOX*, *LIPC*, *TRIB1 and ZFHX3*

Genome-wide single-variant analyses identified five novel amino acid-associated loci at least 1 Mb away from the nearest known GWAS variant (see Table 1 and Supplementary Material, Fig. S6A–E). Of the five novel loci (see Table 1), two were located in the introns of *LOC157273* (near *PPP1R3B*) and *WWOX*. At the *LOC157273/PPP1R3B* locus, intronic variant rs9987289-A was associated with decreased glycine levels (MAF = 17.0%, $\beta = -0.22$, $P = 2.3 \times 10^{-26}$, Supplementary Material, Fig. S6A). This variant was replicated in the NFBC1966 cohort ($\beta = -0.15$, $P = 7.3 \times 10^{-4}$, see Table 1), and was associated with the risk of type 2 diabetes [odds Ratio (OR) = 1.05, $P = 0.02$] and liver disease (OR = 1.33, $P = 4.7 \times 10^{-18}$, see Supplementary Material, Table S6). Within the *WWOX* region, intronic variant rs9937914-G was associated with increased alanine levels (MAF = 1.47%, $\beta = 0.36$, $P = 3.8 \times 10^{-8}$, Supplementary Material, Fig. S6B).

We identified two additional novel loci in regions previously highlighted by GWAS: first, in the region upstream of *LIPC*, a gene implicated in numerous lipid traits including high-density lipoprotein cholesterol (HDL-C) (22), phospholipids (20), and the ratio of isoleucine and serum total cholesterol (serum-c) (10), rs10468017-T was associated with increased alanine levels (MAF = 33.2%, $\beta = 0.09$, $P = 1.5 \times 10^{-8}$, Supplementary Material, Fig. S6C), and is in strong linkage disequilibrium (LD) with rs1532085 (LD $r^2 = 0.66$), a *LIPC* GWAS locus for HDL (23) and ratio of isoleucine and serum-c (10). Its association with increased alanine levels was confirmed in the NFBC1966 cohort ($\beta = 0.08$, $P = 7.7 \times 10^{-3}$, see Table 1). Second, rs28601761-G, for which we report an association with decreased tyrosine levels (MAF = 42.2%, $\beta = -0.09$, $P = 8.8 \times 10^{-9}$, Supplementary Material, Fig. S6D), is in strong LD with rs2954029 (LD $r^2 = 0.71$), a *TRIB1* GWAS variant for low-density lipoprotein cholesterol (LDL-C), triglycerides, and HDL-C levels (23,24).

At the remaining novel locus, rare variant 16: 73326579 was associated with increased alanine levels (MAF = 0.42%, $\beta = 0.76$, $P = 3.6 \times 10^{-9}$). The variant 16: 73326579 is located within 300 kb of both *HCCAT5* and *ZFHX3*, but is not in strong LD (LD $r^2 < 0.60$) with any coding variant observed in the GoT2D study (see Supplementary Material, Fig. S6E). We computed *P* values (P_{ACT}) for the novel variants after correcting for the nine correlated amino acid traits (25). All of these five novel variants remained genome-wide significantly associated even after correcting for the nine amino acid traits ($P_{ACT} < 5.0 \times 10^{-8}$).

Conditional analyses identify independent signals at five known amino acid loci: *GLDC*, *HAL*, *ALDH1L1*, *ADAMTS3* and *GCSH*

We curated 1519 unique known amino acid-associated variants, and then used them as covariates in the genome-wide conditional analyses (see Supplementary Material, Table S7, Materials and Methods). After conditional analyses, we observed 227 unique variant-trait associations ($P_{conditional} < 5 \times 10^{-8}$) (see Supplementary Material, Table S8), whose distributions in genes are presented in Supplementary Material, Table S3. Among these, we identified five novel signals at established amino acid loci distinct from the previously published GWAS variants (see Table 1): *GLDC* p.Q996H, associated with increased glycine (rs138640017-G, MAF = 0.95%, $\beta = 1.35$, $P_{conditional} = 5.8 \times 10^{-40}$); *HAL* p.G283V, associated with increased histidine levels (rs141635447-A, MAF = 0.46%, $\beta = 0.85$, $P_{conditional} = 9.4 \times 10^{-11}$); rs6564825-G, in an intron of *PKD1L2* and 38 kb downstream of *GCSH*, was associated with increased glycine levels (MAF = 11.7%, $\beta = 0.17$, $P_{conditional} = 2.0 \times 10^{-10}$) and nominally but not coincidently associated with expression level of *PKD1L2* (see Supplementary Material, Table S9); rs190671241-G, an intergenic variant near *ADAMTS3*, associated with increased phenylalanine levels (MAF = 1.70%, $\beta = 0.36$, $P_{conditional} = 2.2 \times 10^{-9}$); and rs112981908-G, an intronic variant of the *ALDH1L1* gene associated with decreased glycine levels (MAF = 11.9%, $\beta = -0.14$, $P_{conditional} = 3.1 \times 10^{-10}$). At each locus, we observed low pairwise LD (LD $r^2 < 0.10$) between the novel variant identified in the METSIM data and the previously published GWAS variant(s). Notably, of the five novel signals at established amino acid loci, three replicated in the NFBC1966 data: *GLDC* p.Q996H, associated with increased glycine (rs138640017-G, $\beta = 0.94$, $P = 2.7 \times 10^{-10}$); *HAL* p.G283V, associated with increased histidine levels (rs141635447-A, $\beta = 1.04$, $P = 1.7 \times 10^{-5}$); and *ADAMTS3* upstream variant rs190671241-G, associated with increased phenylalanine levels ($\beta = 0.39$, $P = 1.6 \times 10^{-4}$). Functional work is necessary to determine whether the novel signals represent additional functional variants in genes known to play a role in amino acid metabolism (e.g. *GLDC* and *HAL*), or whether they point to novel mechanisms.

Single-variant associations exhibit an inverse relationship between allele frequency and effect size

To visualize the relationship between allele frequency and effect size of amino acid-associated variants, we plotted the absolute value of effect size estimates versus MAF for all loci associated with amino acid traits in the METSIM study ($P < 5 \times 10^{-8}$) (see Table 1) and fitted a fractional polynomial spline to the data (see Fig. 1). These results demonstrated a strong, inverse relationship between MAF and effect size ($P_{trend} < 0.001$), consistent with past findings for other traits [e.g. (26)]. This relationship is largely driven by variants with MAF < 5%, five of which were newly identified in this study.

Variant associations with amino acid ratios

Prior studies found more genome-wide significant variant associations with amino acid ratios as compared with amino acid traits alone (8,12). We identified 15 220 significant variant-ratio associations (3822 unique variants) from unconditional analyses of the 36 possible ratios among the nine amino acids measured in the METSIM study. These results are presented in Supplementary Material, Table S10 as a reference for other investigators.

Gene-based tests identify novel gene associations with *BCAT2* and *PYCR1*

To determine the joint contribution of the protein-truncating and missense variants of MAF < 1% on amino acid traits, we

Table 1. Novel and known single variants associations with amino acid traits

Lead variant	Trait	Chr: Pos[a]	Variant annotation	METSIM							NFBC1966		
				MAF (%) (allele)	β (SE)	Var.%	P	GWASSNV[b]	GWAS trait	$P_{conditional}$[c]	MAF (%)	β (SE)	P
Novel single-variant associations with amino acid traits													
rs9987289	Gly	8: 9183358	*LOC157273* intronic	17.0 (A)	−0.22 (0.02)	1.31	2.3×10^{-26}	—	—	2.3×10^{-25}	13.5	−0.15 (0.04)	7.3×10^{-4}
16: 73326579[d]	Ala	16: 73326579	267 kb upstream of *ZFHX3*	0.42 (T)	0.76 (0.13)	0.41	3.6×10^{-9}	—	—	1.3×10^{-6}	0.7	−0.27 (0.17)	0.10
rs10468017	Ala	15: 58678512	45 kb upstream of *LIPC*	33.2 (T)	0.09 (0.02)	0.37	1.5×10^{-8}	—	—	1.2×10^{-3}	33.2	0.08 (0.03)	7.7×10^{-3}
rs9937914	Ala	16: 78422354	*WWOX* intronic	1.47 (G)	0.36 (0.07)	0.35	3.8×10^{-8}	—	—	1.4×10^{-7}	1.3	0.14 (0.13)	0.29
rs28601761	Tyr	8: 126500031	49 kb downstream of *TRIB1*	42.2 (G)	−0.09 (0.02)	0.39	8.8×10^{-9}	—	—	1.5×10^{-7}	40.0	−0.02 (0.03)	0.52
Novel single-variant signals at established amino acid loci													
rs138640017	Gly	9: 6533092	*GLDC* Q996H	0.95 (G)	1.35 (0.08)	3.35	3.5×10^{-65}	rs140348140	Gly (16)	5.8×10^{-40}	1.02	0.94 (0.15)	2.7×10^{-10}
rs141635447[d]	His	12: 96374381	*HAL* G283V	0.46 (A)	0.85 (0.12)	0.62	2.5×10^{-13}	rs7954638	His (16)	9.4×10^{-11}	0.35	1.04 (0.24)	1.7×10^{-5}
rs6564825	Gly	16: 81153894	*PKD1L2* intronic	11.7 (G)	0.17 (0.02)	0.57	3.1×10^{-12}	rs74249229	Gly (16)	2.0×10^{-10}	10.4	−0.01 (0.05)	0.87
rs190671241	Phe	4: 73574142	139 kb upstream of *ADAMTS3*	1.70 (G)	0.36 (0.06)	0.42	2.4×10^{-9}	4: 73542640	His/Phe (8)	2.2×10^{-9}	2.10	0.39 (0.10)	1.6×10^{-4}
rs112981908	Gly	3: 125858480	*ALDH1L1* intronic	11.9 (G)	−0.14 (0.02)	0.37	1.8×10^{-8}	rs1107366	Gly (16)	3.1×10^{-10}	e	e	e

Abbreviations: β (SE), estimated regression coefficient and standard error for the minor allele; Chr/Pos, variant chromosome and position based on hg19 build; MAF% (Allele), minor allele frequency (in percent) with minor allele in parentheses; SNV, single-nucleotide variant; Var%, trait variance explained by variant (in percent).

Amino acid abbreviations: Ala, alanine; Gly, glycine; His, histidine; Phe, phenylalanine; Tyr, tyrosine.

[a]Position based on hg19 build.

[b]Lead GWAS SNV within 1 Mb of the identified lead SNV.

[c]$P_{conditional}$ values result from conditional analyses adjusting for known amino acid signals from previous studies (see Supplementary Material, Table S7).

[d]These two variants were directly genotyped for validation and had 100% concordance with the imputed genotype (see text).

[e]This variant was not in the HRC panel used for analyses in the NFBC1966 dataset.

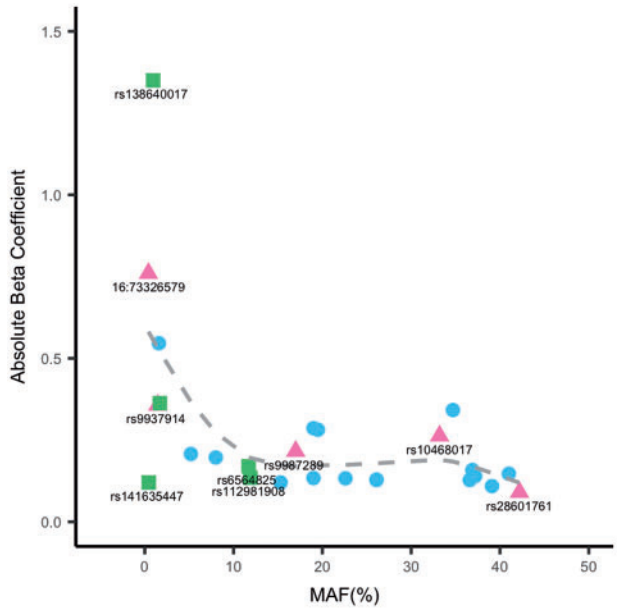

Figure 1. Relationship between minor allele frequency and estimated beta coefficient (β) for loci associated with amino acid levels in the METSIM data. All novel amino acid loci (triangles in pink) are highlighted, in addition to novel signals at known amino acid loci (squares in green) identified through analyses conditioned on all known amino acid GWAS variants. The known amino acid signals are represented with blue circles. The dashed gray line represents a fractional polynomial spline fitted to the data points ($P < 0.001$). β, on the y-axis, is the absolute value of the estimated regression coefficient for a given variant-trait association.

performed gene-based tests (see Supplementary Material, Table S11 and Fig. S7) and applied a significance threshold based on the number of genes tested (~20 000). Given the high correlation among amino acid traits levels (see Supplementary Material, Fig. S2), we did not correct for the number of amino acid traits as a Bonferroni-corrected significance threshold would be overly strict. We identified six gene-trait associations ($P_{gene} < 2.5 \times 10^{-6}$), including four genes previously identified through single-variant association tests: *ALDH1L1* (8) and *GLDC* (16) associated with glycine levels, *HAL* with histidine levels (16) (see Supplementary Material, Table S12), and *DHODH* (previously associated with alanine-to-tyrosine ratio) with alanine levels, as well as two novel associations between *PYCR1* and glycine levels ($P_{gene} = 1.5 \times 10^{-6}$), and *BCAT2* and valine levels ($P_{gene} = 7.4 \times 10^{-7}$, see Table 2).

No missense variants within either *PYCR1* or *BCAT2* achieved genome-wide significance with any amino acid trait in the single-variant association tests, highlighting the utility of gene-based test in novel gene discovery (see Table 2). Despite only suggestive association evidence, the effect of these variants on amino acid trait variance was considerable (the range of absolute β: 0.36–1.02): the carriers of the two missense variants within the gene *PCYR1* exhibited lower mean glycine levels, while the carriers of the three variants within the *BCAT2* gene showed higher mean valine levels, suggesting altering their protein sequences would affect the serum glycine and valine levels, respectively (see Fig. 2).

Discussion

Amino acids are highly heritable traits whose levels have been implicated in the pathogenesis of human complex diseases such as type 2 diabetes (2–4,27,28) and Alzheimer's disease (6). Here, we leveraged dense, experimentally determined and imputed genotypes and report seven novel amino acid associations in the METSIM study that replicated in NFBC1966. Of these associations, two were identified from single-variant testing in the METSIM study and replicated in the NFBC1966 data, and two other loci were identified from gene-based analyses in the METSIM study alone. One of these newly identified variants from single-variant analyses, *LOC157273/PPP1R3B* variant rs9987289-A, also confers increased risk of type 2 diabetes and liver disease. In addition, we fine-mapped known amino acid loci and identified and replicated distinct association signals at three of these loci. Phenotypic variance explained for these nine amino acids by known and novel associations ranged from 10.4% for histidine levels to 28.5% for glycine levels in our data. These results further elucidate the potential mechanisms through which amino acid levels are perturbed, and their potential relationship to disease.

Novel loci highlight a potential role for genes implicated in lipid metabolism and human diseases

Of the five novel amino acid loci highlighted in this study through single-variant analyses, four have previously been implicated in lipid metabolism. First, the rs9987289-A signal near *LOC157273/PPP1R3B*, for which we report an association with decreased glycine levels, has previously been associated with decreased HDL-C, LDL-C and total cholesterol levels (23), as well as increased *PPP1R3B* expression levels in human liver tissue (23). In addition, overexpression of *Ppp1r3b* led to a significant decrease in HDL-C and total cholesterol in a mouse model (23). This variant is also associated with increased risk of type 2 diabetes (29) and liver disease (30). Second, the rs10468017-T signal upstream of *LIPC* associated with increased alanine levels has previously been associated with increased HDL-C (22), altered levels of several circulating phospholipids (20), and the ratio of isoleucine and serum-c (10). Third, the signal near *TRIB1* associated with decreased tyrosine levels has previously been associated with decreased total cholesterol, LDL-C and triglyceride levels (23). Finally, we reported an intronic *WWOX* variant, rs9937914-G, associated with increased alanine levels, and human carriers of predicted loss-of-function variants in *WWOX* were reported to have lower HDL-C (31) levels; in addition, mice lacking *Wwox* exhibit decreased fasting cholesterol, triglyceride and glucose levels (32). These variant associations may represent a secondary effect of altered lipid levels on amino acid metabolism, as previously demonstrated in the setting of obesity (33) and insulin resistance (2). Further work will be required to determine the mechanisms through which these lipid-related loci affect amino acid levels and human complex diseases.

Gene-based tests highlight two novel loci not identified from single-variant testing

PYCR1, which we identified as a gene implicated in glycine levels, encodes a mitochondrial protein involved in biosynthesis of proline and generation of oxidative potential through NADP+ production (34). *PYCR1* was recently identified as the genetic cause of autosomal recessive cutis laxa type 2, highlighting the importance of normal *PYCR1* function in neurodevelopment (35). Functional studies of fibroblasts from affected individuals found increased sensitivity to oxidative stress (36). As redox reactions are critical to amino acid biosynthesis (37), our finding

Table 2. Novel gene-based associations with amino acid traits

Trait	Gene	rsID	Chr: Pos[a]	Annotation	METSIM					NFBC1966		
					MAF (%) (allele)	Genotype counts	β	Variant P	Gene P	MAF (%)	β	Variant P
Novel gene-based associations with amino acid traits												
Gly	*PYCR1*								1.5×10^{-6}	—	—	—
		rs3744807	17: 79890818	G297R	0.13 (T)	8532/22/0	−1.02	1.62×10^{-6}		0.01	1.2	0.23
		rs142225075	17: 79893020	L108V	0.10 (C)	8528/17/0	−0.42	0.08		0.09	−0.5	0.30
Val	*BCAT2*								7.4×10^{-7}	—	—	—
		rs199999090	19: 49299714	R331C	0.18(A)	8515/30/0	0.63	5.36×10^{-4}		—	—	—
		rs117048185	19: 49309776	Q60E	0.59 (C)	8445/100/0	0.36	4.12×10^{-4}		0.71	0.6	0.0003
		rs201148940	19: 49309937	H6R	0.02 (C)	8542/3/0	0.42	0.46		Monomorphic		
Novel gene-based associations with amino acid traits, established association with amino acid ratios												
Ala	*DHODH*[b]								1.36×10^{-7}	—	—	—
		rs201970636	16: 72055088	A195T	0.41 (A)	8475/70/0	0.64	1.94×10^{-7}		0.76	0.3	0.047
		rs201202896	16: 72055187	E228K	0.01 (A)	8544/1/0	−0.80	0.42		—	—	—
		rs199626701	16: 72057113	A290V	0.01 (T)	8544/1/0	1.13	0.26		—	—	—
		rs200181357	16: 72057134	R297H	0.01 (A)	8543/2/0	−0.22	0.76		Monomorphic		
		rs192923495	16: 72057193	R317W	0.09 (T)	8529/16/0	0.26	0.30		0.32	0.0	0.92

Abbreviations: β, estimated regression coefficient for the minor allele; Chr: Pos, variant chromosome and position based on hg19 build; MAF(%), minor allele frequency (in percent) with minor allele in parentheses.

Amino acid abbreviations: Ala, alanine; Gly, glycine; His, histidine; Val, valine.

[a]Position based on hg19 build.

[b]SNPs in *DHODH* previously associated with alanine-to-tyrosine ratio by Kettunen *et al.* (8).

that *PYCR1* missense variants result in decreased glycine levels may suggest reduced oxidative potential *in vivo*.

We also identified an association between *BCAT2* variants and valine levels through gene-based testing. *BCAT2* encodes a mitochondrial enzyme responsible for the first steps in the breakdown of branched chain amino acids (isoleucine, leucine and valine) (37); thus the relationship of *BCAT2* with valine levels is clear. Prior literature reports that the deletion of exon 2 in the mouse homologue of *BCAT2* resulted in a phenotype similar to Maple Syrup Urine Disease (38), an autosomal recessive human inborn error of metabolism characterized by high levels of branched chain amino acids and resulting neurologic symptoms due to the inability to catabolize dietary branched chain amino acids. A human case study of an adult male with mild neurologic symptoms (headaches and mild memory loss) has been reported similar findings, with R170Q and E264K missense variants in *BCAT2* resulting in higher-than-expected levels of leucine, isoleucine and valine (39). Therefore, our finding of *BCAT2* missense variants resulting in increased valine levels is supported by prior literature.

Limitations

Some limitations of this study should be considered. First, our analyses were limited by statistical power secondary to sample size. This was likely one of the contributing factors to our lack of replication of single variant signals in the NFBC1966 data, as the available NFBC1966 replication sample size was modest. Future meta-analyses of amino acid associations are likely to clarify true signals from false positives. Second, our discovery and replication populations of Finnish men (and women for the replication study) limit the generalizability of our findings. However, this study design also provided the genetic homogeneity needed to identify Finnish-ancestry-specific rare variant associations with amino acid traits. Third, our results infer association between genetic markers and amino acid traits; however, elucidating the causal mechanism through which these variants affect amino acid levels will require further functional work.

Summary

These GWAS of nine amino acid traits in 8545 participants of the METSIM study identified five novel single-variant associations, including variant-trait associations near *LOC157273/PPP1R3B*, *WWOX*, *TRIB1*, *LIPC* and *ZFHX3*, of which two were replicated in the NFBC1966 cohort and one (*LOC157273/PPP1R3B*) was also associated with the risk of type 2 diabetes and liver disease. In addition, we identified two novel gene-based signals driven by two and three potentially functional missense variants at *PYCR1* and *BCAT2*, respectively. In *BCAT2*, we validated the association of one rare missense variant in the NFBC1966 study. Our use of a dense reference panel yielded 16.6M genotyped and imputed variants, allowing for high-resolution analyses and fine mapping of independent genetic signals at *GLDC*, *GCSH*, *ALDH1L1*, *ADAMTS3* and *HAL*; of which the signals at *GLDC*, *HAL* and *ADAMTS3* were replicated in the NFBC1966 data. Further work is needed to determine which of the variants identified in this study may affect gene function and the precise roles of the identified genes in amino acid metabolism. These analyses provide further insight into the molecular mechanisms of amino acid metabolism, and, given the importance of amino acid level perturbation in the pathogenesis of numerous human diseases, may yield insights into a wide spectrum of human complex disease.

Materials and Methods

Study participants

Of the 10 197 participants in the METSIM study, we analyzed the subset of 8545 non-diabetic men of mean age 57.3 ± 7.1 years

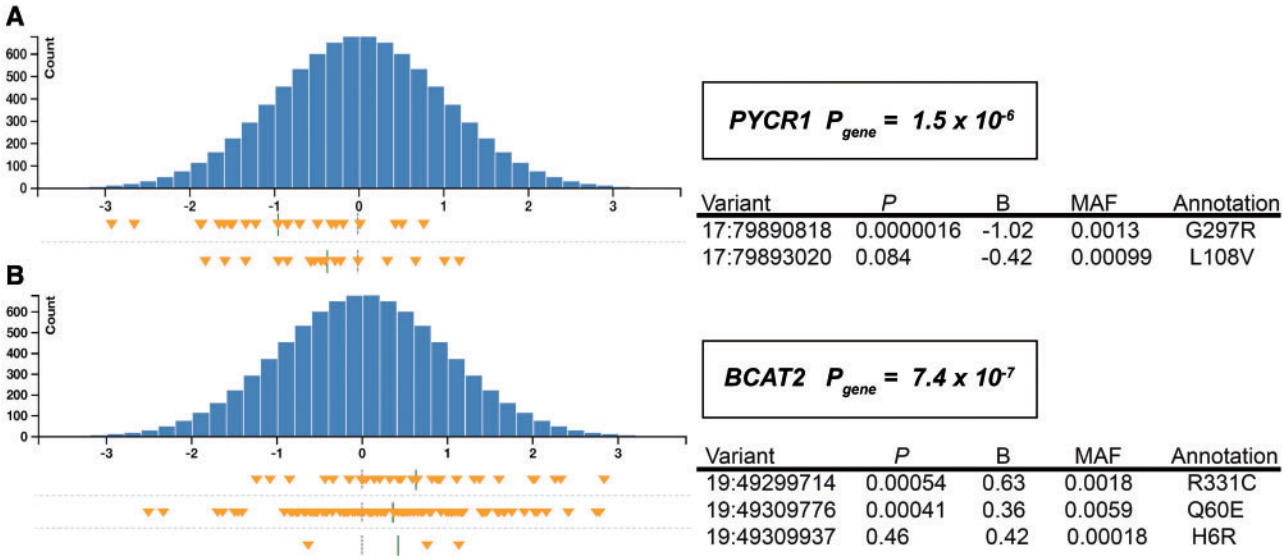

Figure 2. Plots show the trait values of rare variant carriers relative to the distribution of amino acid levels in all individuals. The tables in the right panel show gene-based tests of association with amino acid levels for genes *PYCR1* and *BCAT2*. Histograms show the distribution of the inverse normalized residuals of the trait across all participants for the gene-based test of association at (**A**) *PYCR1* with glycine levels and (**B**) *BCAT2* with valine. The dashed gray line represents the mean inverse normalized residual of trait level for all individuals. The solid black line in each row represents the mean trait level for carriers of each variant. Triangles represent rare variant carriers. The locations of triangles relative to the distribution across all participants indicate the trait levels of rare variant carriers. No individuals were homozygous for the minor allele of any of the listed variants.

and BMI 27.0 ± 4.0 kg/m^2 with NMR amino acid trait measurements (see Supplementary Material, Table S1). Institutional review boards at the University of Kuopio and Kuopio University approved the METSIM study. Written informed consent was obtained from each participant.

Amino acid trait measurement

Blood samples from METSIM participants were obtained and stored in liquid nitrogen until measurement by NMR, as previously described (40). In brief, fasting serum samples collected at enrollment were stored at −80°C and thawed overnight in a refrigerator before sample preparation. A high-throughput serum NMR metabolomics platform was then used to quantify the levels of individual metabolites using a low-molecular weight metabolite data window (1^H NMR spectra) used to identify amino acids (41). We then used iterative lineshape fitting with known chemical shifts to identify and quantify each specific metabolite (42).

We measured nine amino acid levels by NMR spectroscopy: alanine, glutamine, glycine, histidine, isoleucine, leucine, phenylalanine, tyrosine and valine (see Supplementary Material, Table S1). Visualization of the Pearson pairwise correlation matrix between the nine measured amino acid traits was generated using the corrplot package (https://cran.r-project.org/web/packages/corrplot/index.html; date last accessed March 5, 2018) within R (see Supplementary Material, Fig. S2).

Genotyping and imputation

METSIM participant samples were genotyped on the Human OmniExpresss-12v1_C BeadChip (OmniExpress) and Infinium HumanExome-12 v1.0 BeadChip (Exome Chip) platforms. Quality controls included sample-level controls for sex and relatedness confirmation, sample duplication, and detection of sample genetic ancestry outliers using principal component analysis. Based on these quality control measures, we removed 14 samples with sex chromosome anomalies, 18 with evidence of participant duplication, 12 population outliers and 9 samples with non-Mendelian inheritance inconsistencies. In addition, we removed one individual from each of seven monozygotic twin pairs.

We filtered variants with low mapping quality of probes to genome build GRCh37, low genotype completeness (<95% and <98% for the OmniExpress and ExomeChip, respectively), or Hardy-Weinberg equilibrium $P < 10^{-6}$.

We phased OmniExpress variants passing quality control with SHAPEIT v2 (43) and imputed them using minimac v2 (44). For imputation, we used a reference panel of 20.9M variants from the GoT2D study (including SNVs, indels and large deletions) based on the whole genome sequence of 2874 Europeans, including 1004 Finnish individuals—the largest panel of Finnish genomes available (45). Following imputation, variants directly genotyped on the ExomeChip were added. In cases of common markers between imputed and genotyped variants, we used the directly genotyped call from the ExomeChip. The distribution of imputation quality and MAF for each imputed variants are presented in Supplementary Material, Figure S3. We carried forward 16 607 533 variants with high imputation quality (i.e. minimac RSQ ≥ 0.3) for further single-variant association testing.

Single-variant analyses

We performed single-variant association tests on imputed genotype dosages for all variants with a minor allele count ≥ 3. Association tests assumed an additive genotype model and accounted for cryptic relatedness among the Finnish population using the EMMAX linear mixed model approach (46), as implemented in EPACTS (http://genome.sph.umich.edu/wiki/EPACTS; date last accessed March 5, 2018). We adjusted amino acid traits for age, age^2 and BMI, and then inverse normalized the residuals. We applied normalization of trait levels to control for type-I error caused by skewed distributions, although this normalization

may reduce power to discover associated variants. We created association plots for the novel variants using LocusZoom (http://locuszoom.sph.umich.edu/locuszoom/; date last accessed March 5, 2018). In addition, we computed *P*-values (P_{ACT}) for the novel variants after correcting for the nine correlated amino acid traits (25). We used a conventional significance threshold of $P < 5 \times 10^{-8}$ in single-variant association testing.

Replication in the Northern Finnish Birth Cohort

The associations in the novel regions were replicated *in silico* in the Northern part of Finland: The 1966 cohort (the 'Northern Finnish Birth Cohort', or NFBC1966) (47). NFBC1966 is a prospective follow-up study of children from the two northernmost provinces of Finland born in 1966. All individuals still living in northern Finland or the Helsinki area ($n = 8463$) were contacted and invited for clinical examination. A total of 6007 participants attended the clinical examination at the participants' age of 31 years. Among them, 5402 samples were genotyped on Illumina HumanCNV370DUO Analysis BeadChip (48), and were then imputed to the Haplotype Reference Consortium (HRC) reference (49) and 1000 Genomes Project Phase 3 (50) on the Michigan Imputation Server (https://imputationserver.sph.umich.edu/index.html; date last accessed March 5, 2018). The association for the novel region variants and rare variants were looked up in 2591 samples. Given our focused hypothesis, we set a threshold for significance in replication as $P \leq 0.05$.

Associations of novel amino acid SNVs with end-organ phenotypes

We investigated the association of the novel amino acid regions variants with the risk of type 2 diabetes, Alzheimer's diseases and liver disease. For type 2 diabetes, we used publically available data in large-scale Europeans ($N = 159\,208$) from the DIAGRAM consortium (http://www.diagram-consortium.org; date last accessed March 5, 2018) (29). For Alzheimer's disease, we examined associations in the ADGC consortium ($N = 54\,162$) (51). Finally, for liver disease, we used association summary statistics data the GOLD consortium ($N = 7176$) (30). We used proxy single-nucleotide polymorphisms (SNPs) tightly linking with the novel variant if our variant was not available.

Analysis of amino acid trait variance

We estimated the phenotypic variance explained by genetic variants for inverse normalized amino acid traits as described previously through GCTA v1.26 (see Supplementary Material, Fig. S1) (52). We removed 1153 close relatives through kinship cut-off of 0.0075 in KING 1.4 (53), and then estimated the phenotypic variance in 7392 unrelated samples (53). To account for the effect of population structure, we used the top 10 principal components as covariates. In brief, we carried out a primary analysis that consisted of a simultaneous analysis of all 16.6M variants, and a secondary analysis considering only the 2580 variants determined to be genome-wide significant for at least one amino acid trait (see Supplementary Material, Table S2).

Validation of imputed rare variants

We used TaqMan SNP genotyping (Thermo Fisher Scientific) or Sanger sequencing to validate genotypes at three trait-associated ($P < 5 \times 10^{-8}$) and rare imputed variants (MAF < 0.5%) (see Supplementary Material, Table S5). We examined all individuals predicted (on the basis of imputation) to be heterozygous carriers at any of the three sites, as well as additional non-carriers.

Genome-wide conditional analyses

To identify additional independent genetic signals for amino acid traits at known GWAS loci, we conducted a comprehensive genome-wide conditional association analysis. We curated a database of prior published studies of genetic associations with amino acid and related traits to identify distinct variant associations in conditional analyses. To identify published studies, we screened a GWAS catalogue (http://www.ebi.ac.uk/gwas/; date last accessed March 5, 2018), used SNIPPER (https://csg.sph.umich.edu/boehnke/snipper/; date last accessed March 5, 2018) to query publicly available databases for published variants and loci, and performed literature review using PubMed (https://www.ncbi.nlm.nih.gov/pubmed/; date last accessed March 5, 2018) and Google Scholar (https://scholar.google.com; date last accessed March 5, 2018). We focused on proteinogenic amino acid and related traits (e.g. citrate) in European populations (see Supplementary Material, Table S7).

The curated list contained 2615 variants (of which 1519 were unique, with several variants having multiple trait associations) spanning >100 loci from 14 studies (see Supplementary Material, Table S7). These associated variants were then filtered for pairwise LD ($r^2 > 0.95$) to 408 variants (see Supplementary Material, Table S7). For the 2580 amino acid associated variants identified in discovery analyses (see Supplementary Material, Table S2), we performed a secondary analysis conditioning on the LD-pruned list of 408 independent genetic variants. A variant with *P*-value $< 5 \times 10^{-8}$ was considered to be a novel secondary signal within known amino acid traits loci after conditioning on these 408 independent genetic variants.

Amino acid ratio tests of association

Prior investigations of genetic associations with amino acid trait variation have reported extensive findings with amino acid ratios (8,12). While amino acid ratios were not the focus of our investigation, we included discovery analyses with 36 amino acid ratios listed in Supplementary Material, Table S10.

Gene-based tests of association

We performed gene-based tests of association using SKAT-O (21) with EMMAX (46) to determine the joint contribution of protein-truncating (i.e. nonsense, frameshift and essential splice variants) and missense variants with MAF < 1% on amino acid traits, as described in our previous study (54). For these analyses, we included only coding variants directly genotyped on either the OmniExpress or Exome array. Missing genotype data (proportion < 2%) were imputed with variant-specific mean genotype since SKAT-O requires complete data (21). A total of 51 898 protein-truncating or missense variants in 13 996 genes met these criteria (see Supplementary Material, Table S11 for a summary of variant distributions within genes). We considered a gene-based result exome-wide significant at a *P*-value threshold of 2.5×10^{-6} (0.05/20 000) to account for the number of genes in these gene-based analyses.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. A.J.K. and P.S. are shareholders of Brainshake Ltd., a company offering NMR-based metabolite profiling. A.J.K. and P.S. report employment relation for Brainshake Ltd. All other authors report no conflicts of interest.

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