

Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels

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Abstract

Aims/hypothesis Fasting plasma levels of branched-chain amino acids (BCAAs) are associated with insulin resistance, but it remains unclear whether there is a causal relation between the two. We aimed to disentangle the causal relations by performing a Mendelian randomisation study using genetic variants associated with circulating BCAA levels and insulin resistance as instrumental variables.

Methods We measured circulating BCAA levels in blood plasma by NMR spectroscopy in 1,321 individuals from the ADDITION-PRO cohort. We complemented our analyses by using previously published genome-wide association study (GWAS) results from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) ($n=46,186$) and from a GWAS of serum BCAA levels ($n=24,925$). We used a genetic risk score (GRS), calculated using ten established fasting serum insulin associated variants, as an instrumental variable for insulin resistance. A GRS of three variants increasing circulating BCAA levels was used as an instrumental variable for circulating BCAA levels.

Results Fasting plasma BCAA levels were associated with higher HOMA-IR in ADDITION-PRO (β 0.137 [95% CI 0.08, 0.19] $p=6 \times 10^{-7}$). However, the GRS for circulating BCAA levels was not associated with fasting insulin levels or HOMA-IR in ADDITION-PRO (β -0.011 [95% CI -0.053, 0.032] $p=0.6$ and β -0.011 [95% CI -0.054, 0.031] $p=0.6$, respectively) or in GWAS results for HOMA-IR from MAGIC (β for valine-increasing GRS -0.012 [95% CI -0.069, 0.045] $p=0.7$). By contrast, the insulin-resistance-increasing GRS was significantly associated with increased BCAA levels in ADDITION-PRO (β 0.027 [95% CI 0.005, 0.048] $p=0.01$) and in GWAS results for serum BCAA levels (β 1.22 [95% CI 0.71, 1.73] $p=4 \times 10^{-6}$, β 0.96 [95% CI 0.45, 1.47] $p=3 \times 10^{-4}$, and β 0.67 [95% CI 0.16, 1.18] $p=0.01$ for isoleucine, leucine and valine levels, respectively) and instrumental variable analyses in ADDITION-PRO indicated that HOMA-IR is causally related to higher circulating fasting BCAA levels (β 0.73 [95% CI 0.26, 1.19] $p=0.002$).

Conclusions/interpretation Our results suggest that higher BCAA levels do not have a causal effect on insulin resistance while increased insulin resistance drives higher circulating fasting BCAA levels.

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Keywords Branched-chain amino acids · Fasting insulin variants · Genetic risk score · Insulin resistance · Mendelian randomisation

Abbreviations

BCAA Branched-chain amino acid
GRS Genetic risk score
GWAS Genome-wide association study
MAGIC The Meta-Analyses of Glucose and Insulin-related traits Consortium
SNP Single nucleotide polymorphism

Introduction

Insulin resistance is one of the primary and earliest detectable defects of metabolic dysregulation that leads to type 2 diabetes [1]. Multiple epidemiological studies have shown an association between increased fasting levels of circulating branched-chain amino acids (BCAAs) and increased insulin resistance [2]. A recent study provided a potential mechanistic explanation for how higher BCAA levels could cause insulin resistance by promoting lipid accumulation in the muscle through 3-hydroxyisobutyrate, a catabolic intermediate of the BCAA valine [3]. However, other studies indicate that increased circulating BCAA levels may be the consequence of impaired insulin action and merely a marker of increased insulin resistance [2].

Genetic variants identified in genome-wide association studies (GWAS) for circulating BCAA levels and insulin resistance can be utilised as instrumental variables in Mendelian randomisation [4–6], to examine causal relations between the two. So far, four variants have been shown to associate with circulating BCAA levels in the fasting state [5]. The rs9637599 variant associated with circulating BCAA levels is located 2 kb upstream of the *PPMIK* gene, which encodes a mitochondrial phosphatase known to play a vital role in BCAA metabolism [7]. Three other known variants for circulating BCAA levels are rs10211524, located near to the *SLC1A4* gene (encoding solute carrier family 1, member 4), rs7406661 near the *ASGR1* gene (encoding asialoglycoprotein receptor 1), and the missense variant rs1260326 in the *GCKR* gene (encoding glucokinase receptor). GWAS for fasting insulin levels have uncovered 24 loci, of which ten have been robustly associated with insulin resistance [4, 6].

In the present study, we used genetic risk scores (GRSs) of the known variants associated with circulating BCAA levels or insulin resistance as instrumental variables to examine the causal effect of fasting plasma BCAA levels on insulin resistance and vice versa.

Methods

Study population ADDITION-PRO is a longitudinal cohort study of individuals at low to high risk of type 2 diabetes, nested within the population-based ADDITION-Denmark trial of screen-detected diabetes [8, 9]. A total of 1,321 individuals were included in the present study, 1182 individuals were included as high-risk individuals (150 had isolated impaired fasting glucose [IFG]; 133 had isolated impaired glucose tolerance [IGT]; 50 had both IFG and IGT; 849 had high diabetes risk) and 139 were classed as having low diabetes risk. High (≥ 5 points on diabetes risk score) and low (< 5 points) diabetes risk were assessed using a slightly

modified version of the Danish diabetes risk questionnaire, completed by study participants. The questionnaire scored the participants on the basis of their levels of known risk factors for diabetes including age, sex, BMI, known hypertension, family history of type 2 diabetes, gestational diabetes and leisure time physical activity [8]. Characteristics of the study participants are given in electronic supplementary material (ESM) Table 1. All individuals who fasted ≤ 7 h before fasting blood samples were taken, or who used diabetes medication, were excluded. The study was approved by the Ethics Committee of the Central Denmark Region (journal no. 20080229) and was conducted in accordance with the Helsinki Declaration. All participants provided written informed consent.

Anthropometric measurements Body weight, height, and hip and waist circumference were measured as previously described [10]. BMI was calculated as weight (kg) divided by height (m) squared.

Measurement of insulin resistance Fasting plasma glucose and serum insulin measurements have been described previously [10]. HOMA-IR was calculated as fasting glucose (mmol/l) \times [fasting insulin (pmol/l)/6.945]/22.5 [10].

Measurement of fasting plasma BCAAs Fasting blood samples were collected in EDTA tubes and plasma was stored at -80°C . Proton NMR spectroscopy was used to measure amino acid levels. NMR methods have been described in detail previously [5]. Total circulating BCAA levels were defined as the sum of fasting isoleucine, leucine and valine levels.

Genotyping We genotyped 1,657 participants from the ADDITION-PRO cohort using the Illumina Infinium HumanCoreExome Beadchip (Illumina, San Diego, CA, USA). Genotypes were called using the Genotyping module (version 1.9.4) of GenomeStudio software (version 2011.1, Illumina). We excluded individuals who were first degree relatives, duplicates, outliers (ethnic or otherwise) as determined by visual inspection of principal components, or had extreme inbreeding coefficients (by visual inspection of F -statistics), mislabelled sex, or call rate $< 95\%$, leaving 1,348 individuals who passed quality control criteria. A total of 1,321 individuals had information on total circulating BCAA levels, HOMA-IR and fasting serum insulin levels. The quality control of genotype data was based on the guidelines described previously [11]. The genotypes used in the current study were imputed with high quality (IMPUTE2 info > 0.95) into the 1000 genomes phase 1 panel using IMPUTE2 [12, 13]. The imputation quality was high (IMPUTE2 info > 0.95) for all imputed variants included in the current study (four of ten fasting insulin variants

[rs4846565, rs3822072, rs6822892, rs4865796] and three variants for circulating BCAA levels [rs9637599, rs10211524, rs7406661]). All variants included in this study were in Hardy–Weinberg equilibrium ($p > 0.05$).

Instrumental variables To generate an instrumental variable for circulating BCAA levels, we used publicly available GWAS meta-analysis results for isoleucine, leucine and valine levels from up to 24,900 individuals [5]. We calculated an unweighted GRS using the most significant variant associated with circulating BCAA levels in three of the four known BCAA loci (ESM Table 2). We excluded one known locus, in *GCKR*, owing to its known pleiotropic effect on multiple human complex traits [14]. In addition, we constructed a GRS for circulating BCAA levels with and without the *PPMK1* single nucleotide polymorphism (SNP) rs9637599 and performed analysis considering the association of rs9637599 with BMI ($p = 0.035$) [15] and obesity class I ($p = 0.00042$) [16] in the publicly available GWAS summary data.

To generate an instrumental variable for insulin resistance, we calculated an unweighted GRS using the fasting insulin associated variants reported in a GWAS meta-analysis of the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) [4] (ESM Table 3). To improve the specificity of the score, we only included ten fasting serum insulin variants known to be associated with lower HDL-cholesterol and higher triacylglycerol, a biochemical hallmark of insulin resistance [6]. The score was constructed by summing the number of insulin-increasing alleles reported by MAGIC [4].

We complemented our analyses of individual-level data in ADDITION-PRO by testing the associations of valine, leucine and isoleucine GRSs with HOMA-IR using GWAS summary results from MAGIC [4]. Similarly, we tested for the association of fasting insulin GRS with valine, leucine, and isoleucine levels using GWAS summary results by Kettunen et al [5]. We implemented a multi-SNP genotype risk score method (R package ‘gtx’) used previously by Dastani et al [17]. This method approximates the average effect of variants that increase circulating BCAA levels on insulin resistance, and that of insulin-resistance-increasing variants on plasma BCAA levels, in publicly available GWAS meta-analysis results, enabling the estimation of causal effects in large sample sizes [17, 18]. However, the method does not involve a formal instrumental variable analysis. In brief, we identified three SNPs (rs10211524, rs9637599, rs7406661) that were statistically or nominally associated with valine levels, leucine levels, or isoleucine levels (ESM Table 2), to test for the association of valine GRS, leucine GRS or isoleucine GRS with HOMA-IR in GWAS summary results from MAGIC. We removed the variant rs7406661 from the analyses because this SNP was not available in GWAS summary results for HOMA-IR from MAGIC.

Statistical analysis Statistical analyses were performed using R, version 3.1.3 (www.R-project.org/). We removed extreme outliers for circulating BCAA levels (> 4 SD), and normalised the distributions of circulating BCAA levels, HOMA-IR and insulin using rank-based inverse normal transformation. Multiple linear regression analyses were used to test for associations between circulating BCAAs, insulin resistance and genetic variants adjusting for age, sex and BMI. We performed a bi-directional Mendelian randomisation analysis using two-stage least-squares regression to estimate the possible causal relationship between plasma BCAA levels and insulin resistance, and vice versa [19].

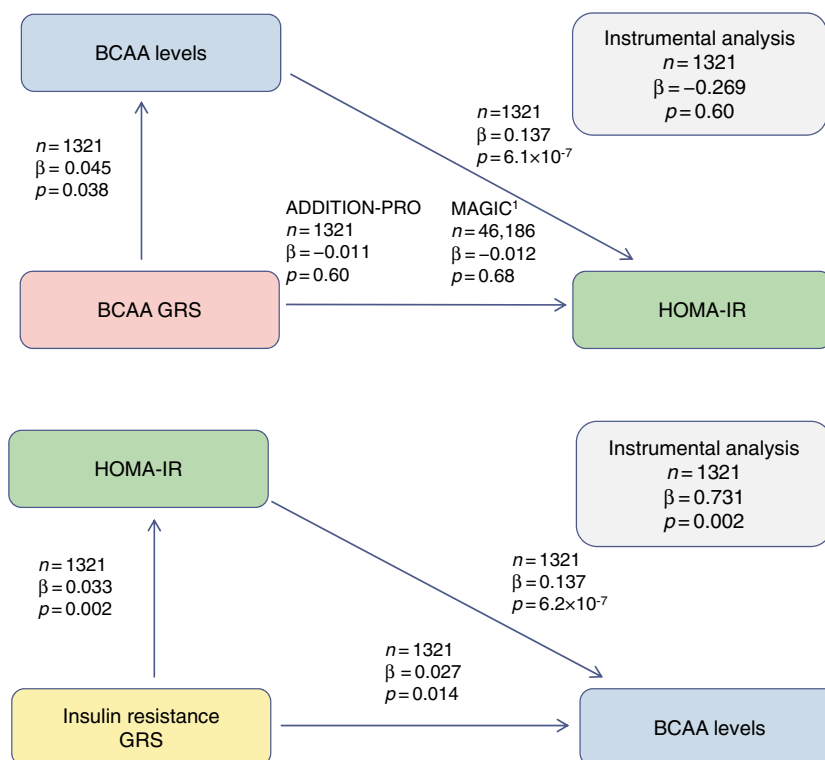
Results

Higher fasting plasma BCAA levels were significantly associated with increased fasting serum insulin levels (β 0.127 [95% CI 0.073, 0.181] $p = 4 \times 10^{-6}$) and HOMA-IR (β 0.137 [95% CI 0.083, 0.190] $p = 6 \times 10^{-7}$) in the ADDITION-PRO cohort (Fig. 1). The GRS for circulating BCAA levels increased plasma BCAA levels by 0.045 SD/allele ([95% CI 0.002, 0.087] $p = 0.04$, $n = 1,321$) but no association was found between the BCAA GRS and fasting insulin levels ($\beta = -0.011$ SD/allele, [95% CI -0.053 , 0.032] $p = 0.6$) or HOMA-IR ($\beta = -0.011$ SD/allele, [95% CI -0.054 , 0.031] $p = 0.6$) (Table 1). Exclusion of individuals belonging to the low diabetes risk group of ADDITION-PRO ($n = 139$) did not change the results (circulating BCAA levels with HOMA-IR, $\beta = 0.160$, $p = 2 \times 10^{-8}$ and circulating BCAA GRS with HOMA-IR, $\beta = -0.021$, $p = 0.4$).

The insulin resistance GRS increased fasting insulin levels by 0.037 SD/allele ([95% CI 0.15, 0.059] $p = 9 \times 10^{-4}$) and HOMA-IR by 0.033 SD/allele in the ADDITION-PRO cohort ([95% CI 0.012, 0.055] $p = 0.002$). The insulin resistance GRS also significantly increased fasting plasma BCAA levels ($\beta = 0.027$ SD/allele, [95% CI 0.005, 0.048] $p = 0.01$), suggesting a causal effect of insulin resistance on circulating BCAA levels (Fig. 1).

To estimate the causal effect of HOMA-IR on plasma BCAA levels and vice versa in ADDITION-PRO, we performed instrumental variable analyses where the GRS for HOMA-IR (F -statistic 10.4) and circulating BCAA levels (F -statistic 4.7) were used as instruments. Consistent with the observational association between HOMA-IR and plasma BCAA levels, genetically instrumented HOMA-IR increase showed a causal effect on circulating BCAA levels in the fasting state; each SD increase in HOMA-IR increased plasma BCAA levels by 0.73 SD ([95% CI 0.26, 1.19] $p = 0.002$). By contrast, a genetically instrumented increase in fasting plasma BCAA levels did not show a significant causal effect on HOMA-IR ($\beta = -0.27$ SD, [95% CI -0.054 , 0.031] $p = 0.6$) (Fig. 1). Excluding the rs9637599 SNP from the GRS for

Fig. 1 Mendelian randomisation study. Fasting plasma amino acid levels and HOMA-IR were inverse-normally transformed and β values (adjusted for age, sex and BMI) are expressed in units of SD of the inverse-normally transformed traits, except for the results from the MAGIC consortium, where the β values are expressed in log transformed units [4]. ¹The results shown are for the valine-increasing GRS



circulating BCAA levels, our results remained unchanged (data not shown).

The lack of association between the GRS for circulating BCAA levels and indices of insulin resistance could be due to the small sample size of ADDITION-PRO. Therefore, we implemented additional GRS analyses using a method that approximates the average effect of variants increasing circulating BCAA levels on insulin resistance, and that of insulin-resistance-increasing variants on circulating BCAA levels, in publicly accessible GWAS meta-analysis results [5, 14] (ESM Table 4). In these analyses, we examined GRSs for the three circulating BCAAs (isoleucine, leucine,

valine) separately rather than combined, because of the lack of availability of GWAS results for combined levels of circulating BCAAs. Despite large sample size ($n = 46,186$), isoleucine-, leucine- and valine-increasing GRSs (rs10211524, rs9637599) were not associated with HOMA-IR ($\beta -0.024$, [95% CI $-0.132, 0.084$] $p = 0.7$, ($\beta -0.019$, [95% CI $-0.097, 0.059$] $p = 0.6$) and $\beta -0.012$, [95% CI $-0.069, 0.045$] $p = 0.7$, respectively). However, on the contrary, the HOMA-IR-increasing GRS was significantly associated with increased isoleucine ($\beta 1.22$, [95% CI $0.71, 1.73$] $p = 4 \times 10^{-6}$, $n = 24,775$), leucine ($\beta 0.96$, [95% CI $0.45, 1.47$] $p = 3 \times 10^{-4}$, $n = 24,728$) and valine ($\beta 0.67$, [95% CI $0.16, 1.18$] $p = 0.01$, $n = 24,900$) levels.

Table 1 Association of the BCAA GRS with fasting glycaemic traits and plasma BCAA levels in the ADDITION-PRO cohort

Variable	β (95% CI)	<i>p</i>
Fasting plasma isoleucine	0.027 (−0.015, 0.069)	0.202
Fasting plasma leucine	0.057 (0.016, 0.098)	0.007
Fasting plasma valine	0.048 (0.003, 0.093)	0.036
Fasting total plasma BCAA	0.045 (0.002, 0.087)	0.038
Fasting plasma glucose	−0.016 (−0.064, 0.032)	0.502
Fasting serum insulin	−0.011 (−0.053, 0.032)	0.615
HOMA-IR	−0.011 (−0.054, 0.031)	0.602

β and 95% CI are expressed in units of SD per BCAA-increasing allele and were calculated from multiple linear regressions adjusted for age, sex and BMI

Discussion

Using a GRS for circulating BCAA levels as an instrumental variable, we found no genetic evidence of a causal effect of circulating BCAA levels on insulin resistance in ADDITION-PRO or in MAGIC GWAS meta-analysis results. By contrast, our analyses using an insulin resistance GRS indicate that insulin resistance has a causal effect on plasma BCAA levels.

Increased plasma BCAA levels are associated with insulin resistance, but the role of circulating BCAAs as a potential causal factor in insulin resistance is unclear [2, 12]. In rats, administration of a mixture of a high fat diet and BCAAs is

accompanied by chronic activation of mammalian target of rapamycin complex 1 (mTORC), p70-S6 kinase 1 and c-Jun N-terminal kinase (JNK), and phosphorylation of IRS1 (Ser307), which contribute to the development of insulin resistance and diabetes [20]. A recent study in mice suggested that elevated BCAA levels could cause insulin resistance by promoting lipid accumulation in the muscle [3]. However, other studies suggest that elevated BCAA levels are just a marker of impaired insulin action and not a causative factor in the development of insulin resistance [2]. Our results from the Mendelian randomisation are in accordance with this hypothesis, indicating that circulating BCAAs have no causal effect on insulin resistance.

Insulin is the chief regulator of amino acid metabolism and changes in fasting BCAA levels may be regulated by the effect of insulin on the rate of appearance and clearance of BCAA together with a decreased activity of catabolic enzymes [2]. Loss-of-function mutations in *PPMIK* in humans [21], and disruption of key BCAA metabolism in obese mice and Zucker rats, exhibit a reduced expression of the mitochondrial isoform of branched chain-amino-acid transaminase (BCAT2; which catalyses the first and reversible step in BCAA catabolism), and mitochondrial branched chain α -keto acid dehydrogenase (BCKD) complex E1- α (which catalyses the rate-controlling and the first irreversible step), leading to increased plasma BCAA levels [22, 23]. A decreased BCAA metabolism in fat tissue may contribute to higher BCAA levels in individuals with insulin-resistant obesity [2, 22–24]. Intriguingly, a recent study also showed that the gut microbiota can contribute to elevated BCAA levels in insulin-resistant states [25].

Mendelian randomisation studies exploit the fact that genotypes are randomly assigned at meiosis in order to study causal relationships between traits without being affected by confounding or reverse causality [19]. The GRS for circulating BCAA levels and HOMA-IR did not show an association with any key covariates in ADDITION-PRO (ESM Table 5). While we attempted to maximise the specificity of the genetic instruments, we cannot fully exclude the possibility of residual pleiotropy, i.e. that the selected genetic variants act not only on circulating BCAA levels or insulin resistance, but also on other metabolic pathways independent of their influence on BCAA levels or insulin resistance. Furthermore, while we were able to study the causal relationship between circulating BCAAs and insulin resistance as such, we were not able to study more detailed biological mechanisms that may mediate the causal link between the two.

In conclusion, our results do not support the hypothesis that plasma BCAA levels have a causal effect on insulin resistance, but indicate that insulin resistance drives higher circulating BCAA levels.

Data availability The genome-wide meta-analysis summary results for HOMA-IR and BCAA are found in (www.magicinvestigators.org/downloads) and (www.computationalmedicine.fi/data#NMR_GWAS), respectively. Other relevant data for the present study are shown in the paper and in the ESM. If you wish to see additional data, the authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data is available at the Novo Nordisk Foundation Center for Basic Metabolic Research, section of Metabolic Genetics whose authors may be contacted via torben.hansen@sund.ku.dk.

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Duality of interest OP and TH hold personal shares in Novo Nordisk A/S; the remaining authors declare no potential conflict of interest relevant to this article.

Contribution statement YM designed the study, analysed and researched data, and wrote the manuscript. AJ contributed NMR data, and reviewed and edited the manuscript. CTH performed imputation and quality control of the genotype data, and reviewed and edited the manuscript. KHA contributed to analysis and interpretation of data, reviewed and edited the manuscript, and contributed to discussion. DRW and MEJ designed the cohort, and reviewed and edited the manuscript. NG contributed to analysis and interpretation of data, and reviewed and edited the manuscript. OP contributed to analysis and interpretation of data, reviewed and edited the manuscript, and contributed to discussion. TOK designed the study, researched data, reviewed, wrote and edited the manuscript and contributed to discussion. TH designed the study, reviewed and edited the manuscript and contributed to discussion. All the authors contributed to the design of the study, interpretation of the data, critical revision of the article, and approved the final draft of the manuscript. TH is the guarantor of this study.

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