

AMPK subunits harbor largely non-overlapping genetic determinants for body fat mass, glucose- and cholesterol metabolism

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Précis

AMPK genes harbor non-overlapping determinants for diabetes-/atherosclerosis-related traits. Summation of body-fat-raising alleles reveals additivity and a marked effect size of the alleles.

Abstract

Context: AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme and central regulator of cellular energy metabolism. The impact of single nucleotide polymorphisms (SNPs) in all seven subunit genes on adiposity, glucose- and lipid metabolism has not been systematically studied yet.

Objective: To analyze the associations of common SNPs in all AMPK genes, and of different scores thereof, with adiposity, insulin sensitivity, insulin secretion, blood glucose, total-, LDL- and HDL-cholesterol and triglycerides.

Study Design and Methods: A cohort of 2789 non-diabetic subjects from the Tübingen Family study of type-2 diabetes, metabolically characterized by oral glucose tolerance test and genotyped by genome-wide SNP array was analyzed.

Results: We identified largely non-overlapping SNP sets across four AMPK genes (*PRKAA1*, *PRKAA2*, *PRKAG2*, *PRKAG3*) associated with adiposity, insulin sensitivity, insulin secretion, blood glucose, total-/LDL-cholesterol or HDL-cholesterol, respectively. A genetic score of body-fat-increasing alleles revealed per-allele effect sizes on BMI of +0.22 kg/m² ($p=2.3 \cdot 10^{-7}$), insulin sensitivity of $-0.12 \cdot 10^{19}$ L²/mol² ($p=9.9 \cdot 10^{-6}$) and 2-h blood glucose of +0.02 mmol/L ($p=0.0048$). Similar effects on blood glucose were observed with scores of insulin-sensitivity-reducing, insulin-secretion-reducing and glucose-raising alleles, respectively. A genetic cholesterol score increased total- and LDL-cholesterol by 1.17 mg/dL per allele ($p=0.0002$ and $p=3.2 \cdot 10^{-5}$, respectively), and a genetic HDL score decreased HDL-cholesterol by 0.32 mg/dL per allele ($p=9.1 \cdot 10^{-6}$).

Conclusions: We describe largely non-overlapping genetic determinants in AMPK genes for diabetes-/atherosclerosis-related traits which reflect the metabolic pathways controlled by the enzyme. Formation of trait-specific genetic scores revealed additivity of allele effects, with body-fat-raising alleles reaching a marked effect size.

Keywords: genetics, energy metabolism, glucose metabolism, lipid metabolism, type-2 diabetes

Introduction

AMP-activated protein kinase (AMPK) is a sensor of intracellular energy load. Upon high intracellular AMP concentrations, the enzyme is allosterically activated and subsequently phosphorylates several substrates, including acetyl-coenzyme-A (CoA) carboxylase, 3-hydroxy-3-methylglutaryl-CoA reductase and glycogen synthase, thereby blocking ATP-consuming biosynthetic pathways and activating ATP-generating catabolic pathways [1]. As a central regulator of energy homeostasis, AMPK controls many metabolic pathways, such as fatty acid synthesis, fatty acid oxidation, cholesterol synthesis, glucose uptake, glycolysis, gluconeogenesis, glycogen synthesis and mitochondriogenesis. Furthermore, AMPK is one of the molecular targets of metformin [2], the first-line drug in type-2 diabetes therapy, and single nucleotide polymorphisms (SNPs) in some of its subunit genes are of potential pharmacogenetic relevance by interfering with treatment response to metformin [3]. AMPK is a heterotrimeric enzyme formed by one catalytic α -subunit, one regulatory β -subunit and one AMP-binding γ -subunit [1]. The α -subunits $\alpha 1$ and $\alpha 2$ are encoded by the genes *PRKAA1* and *PRKAA2*. Furthermore, there are two genes for the β -subunits $\beta 1$ and $\beta 2$, i.e., *PRKAB1* and *PRKAB2*, and three genes for the γ -subunits $\gamma 1$, $\gamma 2$ and $\gamma 3$, i.e., *PRKAG1*, *PRKAG2* and *PRKAG3*. The enzyme's subunit isoform composition depends on tissue-specific expression of the genes.

In contrast to AMPK's well characterized molecular functions in lipid, glucose and energy metabolism, the impact of common genetic variation in its subunit genes on metabolic disorders is less well studied. Single-gene-focused reports described effects of SNPs in *PRKAA2*, *PRKAG2* and *PRKAG3* on cholesterol metabolism [4-6] and of SNPs in *PRKAA1*, *PRKAA2* and *PRKAG2* on type-2 diabetes and type-2 diabetes-related traits (fasting glucose, insulin resistance, diabetic nephropathy, coronary artery disease) in Asian cohorts [6-12]. On the other hand, studies in Pima Indians and Caucasians on selected AMPK subunit genes (*PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG2*) failed to show significant SNP associations with type-2 diabetes and related traits [13-15]. A comprehensive and systematic assessment of common variants in all seven subunit genes has not been reported yet.

Therefore, we analyzed the associations of common SNPs (minor allele frequencies $\geq 5\%$) in all seven AMPK subunit genes, and of different scores thereof, with adiposity, insulin sensitivity/resistance, insulin secretion and plasma concentrations of glucose, total-, low-density-lipoprotein (LDL)- and high-density-lipoprotein (HDL)-cholesterol and triglycerides in 2789 metabolically characterized non-diabetic subjects of the Tübingen Family (TÜF) study of type-2 diabetes who were genotyped on a genome-wide scale with Illumina's Infinium Global Screening Array encompassing about 700,000 SNPs.

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Materials and Methods

Ethics statement. The study protocol was compliant with the ethical principles of the Declaration of Helsinki and approved by the Ethics Board of the Medical Faculty of the Eberhard Karls University Tübingen. All study participants provided written consent to the study.

Study population. A previously described study population of 2789 non-diabetic subjects from Southern Germany derived from the ongoing TÜF study was analyzed [16]. TÜF is recruiting, by interrogation of family members of patients with type-2 diabetes and by study announcements via institutional websites and flyers, subjects at increased risk for type-2 diabetes, i.e., individuals with impaired fasting glycemia and/or body-mass index (BMI) ≥ 27 kg/m² and/or family history of type-2 diabetes and/or previous gestational diabetes [17]. The design of the TÜF study is cross-sectional and thus observational. From all TÜF participants, information on medical history, smoking status and alcohol consumption is being collected, and each participant is undergoing physical examination, routine blood tests and oral glucose tolerance tests (oGTTs). Selection of the study population was based on complete oGTT and genotype data sets.

OGTT. After a 10-h overnight fast, standardized 75-g oGTTs with venous blood sampling at time-points 0, 30, 60, 90 and 120 min were performed as described [17]. In the blood samples, glucose, insulin, C-peptide, triglycerides, total-, LDL- and HDL-cholesterol levels were determined.

Body fat quantification. BMI (in kg/m²) was calculated as weight divided by squared height. Body fat content (in %) was measured by bioelectrical impedance (BIA-101, RJL systems, Detroit, MI, USA). Waist-hip ratio (dimensionless) was calculated as waist circumference (in cm) divided by hip circumference (in cm).

Measurement of carotid intima-media thickness. Intima-media thickness (in mm) of the left and right common carotid arteries was determined by high-resolution ultrasound with a linear ultrasound transducer (10-13 MHz; AU5 Harmonic, Esaote Biomedica, Hallbergmoos, Germany) in B-mode according to the European Mannheim carotid intima-media thickness consensus [18]. The procedure

was recently described in detail [19]. Three replicate measurements were performed for each side, and mean values were calculated for the study participant's left and right carotid arteries. Both mean values were averaged to one mean value of carotid intima-media thickness.

Clinical chemistry. Plasma glucose concentrations (in mmol/L) were quantified with a bedside glucose analyzer (glucose oxidase method, Yellow Springs Instruments, Yellow Springs, OH, USA), plasma triglyceride, total-, LDL- and HDL-cholesterol concentrations (in mg/dL, all) with an ADVIA 1800 clinical chemical analyzer. Serum insulin and C-peptide concentrations (in pmol/L, both) were determined by commercial chemiluminescence assays for ADVIA Centaur (Siemens Medical Solutions, Fernwald, Germany).

Calculations. As a fasting-state-derived insulin resistance index, homeostasis model assessment of insulin resistance (HOMA-IR; in 10^{-6} mol·U/L²) was calculated as $c(\text{glucose}_0) \cdot c(\text{insulin}_0) / 22.5$ with c =concentration and insulin concentrations converted from pmol/L to $\mu\text{U/mL}$ [20]. As an oGTT-derived insulin sensitivity index (ISI; in 10^{19} L²/mol²), the following index proposed by Matsuda and DeFronzo was used [21]: $10,000 / [c(\text{glucose}_0) \cdot c(\text{insulin}_0) \cdot c(\text{glucose}_{\text{mean}}) \cdot c(\text{insulin}_{\text{mean}})]^{1/2}$. Insulin secretion was estimated from the oGTT as area under the curve (AUC) of C-peptide from 0 to 30 min divided by AUC of glucose from 0 to 30 min ($\text{C-peptide}_{\text{AUC0-30}} / \text{glucose}_{\text{AUC0-30}}$; dimensionless $\cdot 10^{-9}$). This index was calculated as $[c(\text{C-peptide}_0) + c(\text{C-peptide}_{30})] / [c(\text{glucose}_0) + c(\text{glucose}_{30})]$.

Selection of SNPs in AMPK genes and generation of trait-specific genetic scores. We screened our genome-wide genotype data from the TÜF study, obtained with the Infinium Global Screening Array (approximately 700,000 SNPs, Illumina, San Diego, CA, USA) [22], for SNPs in the AMPK subunit genes *PRKAA1* (39.0 kb), *PRKAA2* (70.0 kb), *PRKAB1* (13.9 kb), *PRKAB2* (17.4 kb), *PRKAG1* (16.9 kb), *PRKAG2* (321.0 kb) and *PRKAG3* (9.7 kb) and 2 kb of these genes' 5'-flanking regions. The SNPs had to meet the following inclusion criteria: they had to be common (minor allele frequency [MAF] $\geq 5\%$), bi-allelic, successfully genotyped (genotyping success rates $\geq 97\%$), non-linked ($r^2 < 0.8$) and in Hardy-Weinberg equilibrium ($p \geq 0.05$). Based on these criteria, we had to exclude 87 uncommon SNPs, 14

monoallelic SNPs, eight linked SNPs and 11 SNPs not in Hardy-Weinberg equilibrium. On the other hand, we were able to select one SNP in *PRKAA1*, six SNPs in *PRKAA2*, one SNP in *PRKAB1*, two SNPs in *PRKAB2*, no SNP in *PRKAG1*, 77 SNPs in *PRKAG2* and one SNP in *PRKAG3* meeting the inclusion criteria. Thus, a total of 88 SNPs was selected and subjected to single-SNP association analysis (Supplementary Table 1 in [23]). With respect to SNP call rates for individuals, the lowest call rate was 44 SNPs (one individual), the highest was 88 SNPs (86 % of the individuals), and 99 % of the individuals had SNP call rates of >95 %. All metabolic-risk-increasing alleles of SNPs that were at least nominally ($p < 0.05$) associated with the respective trait(s) were summed up to an unweighted trait-specific genetic score.

Statistical analyses. Hardy-Weinberg equilibrium of genotypes was tested by chi-square tests (with one degree of freedom). To ensure normal distribution of continuous variables, all quantitative traits were inverse-normal transformed (INT) by using the formula $\text{INT}(\text{trait}) = \Phi^{-1}\{(\text{rank}[\text{trait}] - 0.5)/n\}$ with Φ^{-1} representing the probit function. For genotype-phenotype association analysis, multiple linear regression models were run based on the standard-least-squares method. In these regression models, the anthropometric/metabolic trait of interest was chosen as dependent variable, the SNP genotype (in the additive inheritance model) or the genetic score as independent variable and gender, age, BMI and ISI as confounding variables whenever appropriate. In the single-SNP analyses, 88 SNPs were tested in parallel. To minimize in this multiple-testing setting the incidence of false-positive results (statistical type-I errors), Bonferroni-correction for the number of tested SNPs was applied and a p-value < 0.00058 was chosen as significance threshold. To calculate the Bonferroni-corrected α -level, the following formula was used: $\alpha_{\text{corrected}} = 1.00 - 0.95^{1/N}$ with N representing the number of SNPs. We did not correct for the different anthropometric/metabolic traits tested as these were not absolutely independent. A p-value < 0.05 was considered as significance threshold when testing the association of a genetic score with a trait. In the multiple linear regression models, the study (N=2789) was sufficiently powered ($1 - \beta \geq 0.8$, two-sided $p < 0.05$) to detect SNP effects on BMI

≥ 1.6 % taking into account the chosen MAF threshold ≥ 5 %. The software package JMP 13.0.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

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Results

Study population. The characteristics of the study population were previously reported [16]. In brief, the Caucasian population at risk for type-2 diabetes had a mean age of 43 (± 14 , standard deviation) years, a mean BMI of 31.0 (± 9.3) kg/m², mean fasting plasma glucose concentrations of 5.24 (± 0.56) mmol/L and 2-h glucose concentrations of 6.41 (± 1.61) mmol/L. The mean plasma concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were 194 (± 38), 118 (± 33), 53.7 (± 14.2) and 123 (± 109) mg/dL, respectively.

Single-SNP analyses. First, we analyzed all 88 common, bi-allelic, array-derived SNPs in *PRKAA1*, *PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG2* and *PRKAG3* for associations with parameters of adiposity (BMI, body fat, waist-hip ratio), insulin sensitivity/resistance (ISI, HOMA-IR) and insulin secretion (C-peptide_{AUC0-30}/glucose_{AUC0-30}) and with plasma concentrations of glucose (glucose₀, glucose₁₂₀), cholesterol (total-, LDL-, HDL-cholesterol) and triglycerides on the single-SNP level (Supplementary Table 1 in [23]). Whenever appropriate, the confounding variables gender, age, BMI and ISI were included in the multiple linear regression models (Supplementary Table 1 in [23]). After Bonferroni correction for multiple testing, we identified 14 SNPs nominally associated ($0.00058 \leq p < 0.05$) with adiposity, nine SNPs nominally associated with insulin sensitivity/resistance ($0.0071 \leq p < 0.05$), five SNPs nominally associated with insulin secretion ($0.0029 \leq p < 0.05$), seven SNPs nominally ($0.0133 \leq p < 0.05$) and one SNP significantly (*PRKAG2* rs56037571; glucose₀, $p = 0.0001$) associated with plasma glucose concentrations, 10 SNPs nominally associated with total-/LDL-cholesterol ($0.0046 \leq p < 0.05$), nine SNPs nominally associated with HDL-cholesterol ($0.0007 \leq p < 0.05$) and three SNPs nominally associated with plasma triglycerides ($0.0295 \leq p < 0.05$; Supplementary Table 1 in [23]).

With the exception of the insulin sensitivity/resistance SNPs, the SNPs associated with a trait were spread across two subunit genes including the largest gene *PRKAG2* (Supplementary Table 1 in [23]). The three SNPs in *PRKAB1* and *PRKAB2* did not show any associations with the tested traits (Supplementary Table 1 in [23]). Interestingly, the trait-specific SNP sets revealed only very limited

overlaps (Figure 1A). This is also reflected by the SNP distribution in the largest AMPK subunit gene *PRKAG2* (321 kb, 37 array-derived SNPs; Figure 1B).

To see whether the SNP associations with BMI were weight- or height-driven, we separately investigated their associations with weight and height (adjusted for gender and age). None of the 10 BMI SNPs was nominally or significantly associated with height ($p \geq 0.08$; Supplementary Table 2 in [23]). By contrast, five of the 10 SNPs showed nominal associations ($p < 0.05$) and four SNPs at least trends for association ($p \leq 0.1$) with weight (Supplementary Table 2 in [23]).

To test whether the SNP associations with waist-hip ratio were waist- or hip-driven, we separately investigated their associations with waist- and hip circumference (adjusted for gender and age). Only one of the eight waist-hip ratio SNPs was nominally associated with both waist- and hip circumference (*PRKAG2* rs9632641; $p = 0.0020$ and $p = 0.0095$, respectively; Supplementary Table 2 in [23]). No other SNP was nominally or significantly associated with hip circumference ($p \geq 0.1$; Supplementary Table 2 in [23]). By contrast, apart from rs9632641, four other SNPs showed nominal association ($p < 0.05$) and two other SNPs at least trends for association ($p = 0.06$, both) with waist circumference (Supplementary Table 2 in [23]).

Trait-specific genetic AMPK scores and their effects. Based on the nominal and significant results of the single-SNP analyses, we generated six trait-specific AMPK scores by summing up the metabolic-risk-increasing alleles, i.e., adiposity-increasing, insulin-sensitivity-reducing, insulin-secretion-reducing, glucose-increasing, total-/LDL-cholesterol-increasing- and HDL-cholesterol-reducing alleles, respectively. Table 1 provides information about the SNPs included in the different genetic scores. Due to the low number of SNPs (three only), we did not generate a plasma-triglyceride-specific score. The AMPK body fat score including the 14 SNPs associated with BMI and/or body fat content and/or waist-hip ratio (allele distribution shown in Figure 2A) revealed a marked and robust effect on BMI adjusted for gender and age: each allele increased BMI by $+0.22 \pm 0.05$ kg/m² (estimate \pm SE, $p = 2.3 \cdot 10^{-7}$; Figures 2A and 2B). Moreover, the score adjusted for gender and age was negatively associated

with insulin sensitivity (ISI, $-0.12 \pm 0.04 \cdot 10^{19} \text{ L}^2/\text{mol}^2$, $p=9.9 \cdot 10^{-6}$; HOMA-IR, $+0.06 \pm 0.02 \cdot 10^{-6} \text{ mol} \cdot \text{U}/\text{L}^2$, $p=4.7 \cdot 10^{-7}$; Figure 2C and Table 2) and positively with plasma glucose concentrations (glucose₀, $+0.005 \pm 0.003 \text{ mmol}/\text{L}$, $p=0.0149$; glucose₁₂₀, $+0.02 \pm 0.01 \text{ mmol}/\text{L}$, $p=0.0048$; Table 2, Figure 2D), and the associations with plasma glucose were rendered non-significant upon additional adjustment for insulin sensitivity (Table 2).

Both, the AMPK insulin resistance score (9 SNPs) and the AMPK insulin secretion score (5 SNPs) robustly impairing insulin sensitivity (ISI, $-0.14 \pm 0.06 \cdot 10^{19} \text{ L}^2/\text{mol}^2$, $p=1.2 \cdot 10^{-5}$; HOMA-IR, $+0.07 \pm 0.02 \cdot 10^{-6} \text{ mol} \cdot \text{U}/\text{L}^2$, $p=1.1 \cdot 10^{-6}$) and insulin secretion ($-3.2 \pm 0.9 \cdot 10^{-9}$, $p=3.4 \cdot 10^{-5}$), respectively, increased fasting plasma glucose concentrations by $+0.01 \pm 0.004 \text{ mmol}/\text{L}$ and $+0.01 \pm 0.007 \text{ mmol}/\text{L}$ per allele ($p=0.0001$ and $p=0.0008$, respectively, adjusted for gender and age; Table 3). Only the insulin secretion score showed an association with 2-h plasma glucose concentrations ($+0.05 \pm 0.02 \text{ mmol}/\text{L}$ per allele, $p=0.0008$, adjusted for gender and age; Table 3).

As expected, the AMPK glucose score (8 SNPs), adjusted for gender and age, was associated with fasting and 2-h plasma glucose concentrations (glucose₀, $+0.02 \pm 0.005 \text{ mmol}/\text{L}$ per allele, $p=1.4 \cdot 10^{-7}$; glucose₁₂₀, $+0.07 \pm 0.02 \text{ mmol}/\text{L}$, $p=2.2 \cdot 10^{-7}$; Table 3).

After adjustment for gender and age, the AMPK cholesterol score, including 10 total-cholesterol- and/or LDL-cholesterol-increasing SNPs, increased both total- and LDL-cholesterol plasma concentrations by $+1.17 \pm 0.29 \text{ mg}/\text{dL}$ and $+1.17 \pm 0.26 \text{ mg}/\text{dL}$ per allele ($p=0.0002$ and $p=3.2 \cdot 10^{-5}$; Figures 3A and 3B). The adjusted AMPK HDL score (9 SNPs) reduced plasma HDL concentrations by $-0.32 \pm 0.09 \text{ mg}/\text{dL}$ per allele ($p=9.1 \cdot 10^{-6}$; Figure 3C). The allele distributions of both scores are shown in Figure 3D.

Associations of the AMPK cholesterol- and HDL scores with carotid intima-media thickness. In a subgroup of 582 genotyped individuals who underwent ultrasound assessment of intima-media thickness of the left and right carotid arteries, the AMPK cholesterol score adjusted for gender and age showed a trend towards positive association with the mean carotid intima-media thickness

(+0.0022±0.0017 mm, $p=0.0531$). By contrast, the AMPK HDL score was not associated with the mean carotid intima-media thickness ($p=0.2$).

Associations of score-derived SNPs with anthropometric and metabolic traits in the Type-2 Diabetes Knowledge Portal. To replicate our single-SNP results, we screened publicly available genome-wide association data deposited in the Type-2 Diabetes Knowledge Portal (<http://www.type2diabetesgenetics.org/>) and looked for associations of the 43 SNPs included in the six genetic scores with their respective traits.

Eight of the 14 AMPK body fat SNPs showed associations with BMI and/or waist circumference and/or waist-hip ratio and/or visceral adipose tissue volume and/or visceral adipose tissue attenuation ($3.5 \cdot 10^{-4} \leq p < 0.05$; Supplementary Table 3 in [23]). Six SNPs from the body fat score were associated with at least one out of nine different measures of insulin sensitivity/resistance ($0.001 \leq p < 0.05$), seven SNPs with hemoglobin A1c (HbA1c) and/or glucose measurements ($6.5 \cdot 10^{-5} \leq p < 0.05$) and four SNPs with type-2 diabetes ($0.001 \leq p < 0.05$; Supplementary Table 3 in [23]).

With respect to the AMPK insulin resistance score, three of the nine SNPs were associated with fasting insulin concentrations, a crude measure of insulin resistance ($0.001 \leq p < 0.05$), three with plasma glucose and/or HbA1c ($9.7 \cdot 10^{-5} \leq p < 0.05$) and one with type-2 diabetes (*PRKAG2* rs7801616, $p=0.046$; Supplementary Table 3 in [23]). Of the five SNPs of the AMPK insulin secretion score, none was associated with any of the reported measures of insulin secretion, four were associated with plasma glucose and/or HbA1c ($0.006 \leq p < 0.05$) and three with type-2 diabetes ($0.001 \leq p < 0.05$; Supplementary Table 3 in [23]).

Among the eight SNPs derived from the AMPK glucose score, four were associated with plasma glucose concentrations ($9.7 \cdot 10^{-5} \leq p < 0.05$), none with HbA1c and five with type-2 diabetes ($0.001 \leq p < 0.05$; Supplementary Table 3 in [23]).

Finally, among the 10 SNPs summed up in the AMPK cholesterol score, one was associated with total cholesterol (*PRKAG3* rs33985460, $p=0.011$) and none with LDL-cholesterol (Supplementary Table 3 in

[23]). Of the nine SNPs of the AMPK HDL score, none was associated with HDL-cholesterol in the publically available data (Supplementary Table 3 in [23]).

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Discussion

Studying common non-linked SNPs in the seven AMPK subunit genes, we identified largely non-overlapping determinants (SNP sets) for adiposity, insulin sensitivity/resistance, insulin secretion, plasma glucose, total-/LDL-cholesterol, HDL-cholesterol and triglycerides, respectively, reflecting the well-described multifaceted regulatory roles of this metabolically important enzyme. Thus, SNP effects on adiposity, plasma triglyceride levels, insulin resistance (e.g., via ectopic lipid deposition) and insulin secretion (e.g., via lipotoxic effects of fatty acids on pancreatic β -cells) could be related to the enzyme's role in the regulation of fatty acid metabolism, and SNP effects on plasma cholesterol levels could very plausibly be explained by the enzyme's regulatory role in cholesterol biosynthesis.

Apart from the insulin-sensitivity-modulating SNPs which were exclusively located in *PRKAG2*, the above mentioned genetic determinants were spread across two subunit genes with *PRKAA2* and *PRKAG2* being crucial for adiposity, triglycerides and HDL-cholesterol, *PRKAA1* and *PRKAG2* for insulin secretion and plasma glucose concentrations and *PRKAG2* and *PRKAG3* for plasma total-/LDL-cholesterol concentrations. This genetic pattern, however, does not necessarily reflect tissue-specific expression of the subunits: for instance, *PRKAA2* and *PRKAG2*, both crucial for adiposity, are according to the GTEx Portal (<https://www.gtexportal.org>) expressed at markedly lower levels than *PRKAA1* and *PRKAG1* in adipose tissue and hypothalamus, two major sites of body fat control; and *PRKAG3* which turned out to be important for plasma total-/LDL-cholesterol concentrations is nearly exclusively expressed in skeletal muscle and is at the detection limit in liver, the predominant LDL production site. This discrepancy may have many different reasons. Amongst others: (i) the SNPs tested may not represent causal variants, but may be in linkage with causal variants outside these gene loci; (ii) if causal, the SNPs may not affect the genes in which they are located, but may alter enhancer/silencer sequences affecting the expression of nearby or more distant genes; and (iii) even though expressed in markedly lower amounts compared to other subunits, causal SNPs affecting the function and/or expression of these less abundant subunits may still exert biologically relevant effects.

The observation that *PRKAG2* SNPs are represented in all genetic scores whereas *PRKAB1* and *PRKAB2* SNPs are not represented in any score may simply be due to the marked differences in gene size (321 kb [*PRKAG2*] versus 13.9 and 17.4 kb [*PRKAB1* and *PRKAB2*, respectively]) and consequently in the absolute number of common SNPs in these genes fulfilling our inclusion criteria (77 [*PRKAG2*] versus one and two [*PRKAB1* and *PRKAB2*, respectively]). As to *PRKAG1* (16.9 kb), we did not identify any common SNP on the array.

Another finding of this genetic study is that summing up the SNPs associated with certain traits to trait-specific genetic scores revealed additivity of AMPK SNP effects resulting, at least in the case of the AMPK body fat score, in an impressive effect size. Thus, having five adiposity-increasing AMPK alleles more than other individuals, e.g., having 13 alleles (eight % of the study population) versus eight alleles (eight % of the study population; Figure 2A), increases the mean BMI by 1.1 kg/m². This means that individuals of 1.75 m height differing by five alleles differ in weight by 3.4 kg on average. For comparison, individuals homozygous for the BMI-raising allele of the most cited obesity SNP in the *FTO* gene (rs9939609; 16 % of the formerly reported study population) were also shown to weigh, on average, about 3 kg more than individuals homozygous for the non-risk allele (32 % of the study population) [24].

In agreement with the well-known associations of obesity with insulin resistance, glucose intolerance and type-2 diabetes and with similar findings obtained with the genetic variants in the major body fat gene *FTO* [25,26], the AMPK body fat score was negatively associated with insulin sensitivity and positively with plasma glucose concentrations. This observation suggests a pathophysiological role of the SNPs incorporated in this score. The finding that the score's association with plasma glucose was abolished by adjustment for insulin sensitivity furthermore provides evidence that the score's effect on glucose tolerance is mediated by its adverse effect on insulin sensitivity. Likewise, the AMPK insulin resistance score and the AMPK insulin secretion score were associated with plasma glucose concentrations and thus appear pathophysiological meaningful as well.

Interestingly, among the eight SNPs of the AMPK glucose score, two were associated with insulin secretion (*PRKAA1* rs249429, *PRKAG2* rs4726101), two with insulin resistance, but not with adiposity (*PRKAG2* rs7801616 and rs55728136), one with adiposity, but not with insulin resistance (*PRKAG2* rs4726101), one with plasma cholesterol (*PRKAG2* rs6950343), and three SNPs, including the only study-wide significant SNP (*PRKAG2* rs56037571), revealed no associations with any other trait. This unexpected finding does not only leave us behind with no clear hints of a unifying pathomechanism of the SNPs summed up in this score, but may rather reflect the fact that multiple, very different pathways can lead to glucose intolerance including, for instance, primary SNP effects on hepatic glucose production. Endogenous glucose production was, however, not assessed in this study.

We tried to replicate our single-SNP findings by interrogating the Type-2 Diabetes Knowledge Portal for associations of the score-derived SNPs with metabolic traits. With respect to associations with the score-defining traits, we could find replication for several SNPs of the AMPK body fat-, insulin resistance- and glucose scores. However, we could not replicate the associations of the SNPs summed up in the AMPK insulin secretion- and HDL scores with their respective traits. The observation that none of the insulin-secretion-score-derived SNPs was associated with the insulin secretion indices deposited in the Type-2 Diabetes Knowledge Portal could be related to the fact that our insulin secretion index was C-peptide-based whereas those of the Portal were exclusively insulin-based. In contrast to serum C-peptide concentrations, serum insulin concentrations are not only determined by insulin secretion but also by insulin clearance [27]. Nevertheless, the insulin secretion SNPs identified in our study are probably of clinical relevance as some of these SNPs were associated with plasma glucose/HbA1c concentrations and/or type-2 diabetes in the Portal's data. We currently have no explanation why we failed to replicate our HDL-cholesterol SNP findings in the Type-2 Diabetes Knowledge Portal, and further investigations are certainly needed to assess these SNPs' role in HDL-cholesterol metabolism.

Screening the literature (PubMed [<https://www.ncbi.nlm.nih.gov/pubmed>] and publications listed in the dbSNP database [<https://www.ncbi.nlm.nih.gov/snp/>]) to find further support for metabolic roles

of the SNPs identified in our study revealed that eight SNPs among the 43 SNPs listed in Table 1 were tested for association with type-2 diabetes, obesity and related traits in genome-wide and hypothesis-driven settings, and only two of them were found to reach study-wide significance. *PRKAA1* rs249429, associated with insulin secretion and blood glucose in our study, was shown in the Diabetes Prevention Program to increase type-2 diabetes incidence in the placebo arm (N=1000), but not in the metformin arm (N=990) [3]. And *PRKAA2* rs10789038, associated with HDL-cholesterol in our study, revealed significant associations in two smaller studies: with type-2 diabetes in a Chinese Han population (406 cases and 214 controls) [7] and with antipsychotic-induced weight gain in 208 patients with schizophrenia [28].

We finally acknowledge the following limitations of the study. First, we only assessed common SNPs depicted on Illumina's Infinium Global Screening Array. Other common SNPs not depicted on the array as well as low-frequency- and rare SNPs that we excluded for statistical power reasons were not analyzed and thus could still include unidentified metabolically relevant variants. And second, all but one SNPs incorporated in trait-specific scores were only nominally associated with the respective traits. Thus, the genetic scores could include SNPs that were associated with the traits just by chance. Therefore, further studies are needed to identify statistical type-I errors and to refine the scores.

In conclusion, we describe largely non-overlapping genetic determinants in AMPK genes for diabetes- and atherosclerosis-related traits which reflect the various metabolic pathways controlled by the enzyme. Thus, our study adds new genetic information that may help decipher AMPK's pleiotropic effects in metabolism. Furthermore, generation of trait-specific genetic scores revealed additivity of allele effects, with body-fat-raising alleles reaching a marked effect size. This emphasizes the hitherto underappreciated relevance of SNP-SNP interactions, compared to single-SNP effects, in genotype-phenotype associations.

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Figure Legends

Figure 1. AMPK SNPs associated with anthropometric and/or metabolic traits. (A) Scheme of SNPs associated with the different anthropometric/metabolic traits. The size of a cycle corresponds to the number of SNPs associated with the trait. Figures above connecting lines represent the numbers of SNPs shared by two traits. A1, *PRKAA1*; A2, *PRKAA2*; G2, *PRKAG2*; G3, *PRKAG3*. (B) Distribution of trait-associated SNPs within the *PRKAG2* gene. The exon-intron structure of the gene is illustrated by upright squares (exons) and exon-connecting zigzag lines (introns). The localization of regulatory features (promoters, enhancers, transcription-factor-binding sites, etc.) is given by colored boxes. SNP positions are indicated by colored asterisks. AMPK, AMP-activated protein kinase; HDL, high-density lipoprotein; SNP, single nucleotide polymorphism; TG, triglycerides

Figure 2. Allele distribution and associations of the AMPK body fat score. The score was formed by summing up all BMI- and/or body-fat- and/or waist-hip-ratio-increasing alleles. (A) Allele distribution of the score and differences in BMI and 2-h plasma glucose concentrations between carriers of 13 and carriers of eight alleles (each group includes eight percent of the study population). Associations of the score with BMI (B), insulin sensitivity (C) and 2-h plasma glucose concentrations (D) are illustrated by regression lines (solid lines) and lines highlighting the 95-% confidence intervals of individual data (dashed lines). Statistical data are derived from multiple linear regression models adjusted for gender and age. AMPK, AMP-activated protein kinase; BMI, body mass index; ISI, insulin sensitivity index

Figure 3. Associations and allele distributions of the AMPK cholesterol score and the AMPK HDL score. The AMPK cholesterol score was formed by summing up all total- and/or LDL-cholesterol-increasing alleles, the AMPK HDL score by summing up all HDL-cholesterol-reducing alleles. Associations of the AMPK cholesterol score with total cholesterol (A) and LDL-cholesterol (B) as well as associations of the AMPK HDL score with HDL-cholesterol (C) are illustrated by regression lines (solid lines) and lines highlighting the 95-% confidence intervals of individual data (dashed lines). Statistical data are derived from multiple linear regression models adjusted for gender and age. (D)

Allele distributions of the AMPK cholesterol score (upper panel) and the AMPK HDL score (lower panel). AMPK, AMP-activated protein kinase; HDL, high-density lipoprotein; LDL, low-density lipoprotein

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Table 1. SNPs included in the different AMPK SNP scores

Gene	SNP	Chr.	Position (GRCh37)	MAF	Body fat score	Insulin resistance score	Insulin secretion score	Glucose score	Cholesterol score	HDL score
PRKAA1	rs249429	5	40782239	0.313			x	x		
PRKAA2	rs11206887	1	57116909	0.170	x					
PRKAA2	rs10789038	1	57117117	0.493						x
PRKAA2	rs11809287	1	57150112	0.058	x					x
PRKAA2	rs932447	1	57170229	0.465						x
PRKAA2	rs4912411	1	57177244	0.337						x
PRKAG2	rs5017429	7	151255160	0.241						x
PRKAG2	rs17173197	7	151268920	0.383						x
PRKAG2	rs7791529	7	151272464	0.160		x				
PRKAG2	rs2538039	7	151283664	0.432						x
PRKAG2	rs2374229	7	151311494	0.267						x
PRKAG2	rs4726070	7	151328218	0.406				x		
PRKAG2	rs6964957	7	151330390	0.385		x				
PRKAG2	rs4726075	7	151335260	0.136		x			x	
PRKAG2	rs3109944	7	151338886	0.130					x	
PRKAG2	rs1105842	7	151364264	0.147	x				x	
PRKAG2	rs56328002	7	151365322	0.121					x	
PRKAG2	rs6978142	7	151378505	0.363	x					
PRKAG2	rs6951177	7	151380597	0.349	x					
PRKAG2	rs1860746	7	151385353	0.174	x					
PRKAG2	rs10277859	7	151387197	0.187	x					
PRKAG2	rs2536085	7	151387531	0.325						x
PRKAG2	rs62478181	7	151390467	0.082	x					
PRKAG2	rs112810819	7	151393354	0.256	x					
PRKAG2	rs75270413	7	151394360	0.197	x					
PRKAG2	rs56765232	7	151394743	0.449	x					
PRKAG2	rs6967507	7	151397487	0.308					x	
PRKAG2	rs4726086	7	151421215	0.490		x			x	
PRKAG2	rs28563985	7	151434444	0.388		x			x	
PRKAG2	rs10278273	7	151440162	0.113					x	
PRKAG2	rs11771330	7	151442744	0.459	x					
PRKAG2	rs11773668	7	151446605	0.236		x				
PRKAG2	rs7801616	7	151452628	0.457		x		x		
PRKAG2	rs9632641	7	151475924	0.189	x		x			
PRKAG2	rs78667038	7	151476195	0.052		x				
PRKAG2	rs7782177	7	151480718	0.301			x			
PRKAG2	rs56037571	7	151492705	0.164				x		
PRKAG2	rs4442045	7	151503301	0.190			x			
PRKAG2	rs62478243	7	151504100	0.176				x		
PRKAG2	rs4726101	7	151513282	0.495	x		x	x		
PRKAG2	rs55728136	7	151517733	0.374		x		x		
PRKAG2	rs6950343	7	151536181	0.079				x	x	
PRKAG3	rs33985460	2	219691801	0.065					x	

AMPK, AMP-activated protein kinase; chr, chromosome; MAF, minor allele frequency; SNP, single nucleotide polymorphism

Table 2. Associations of the AMPK body fat score with anthropometric and metabolic traits

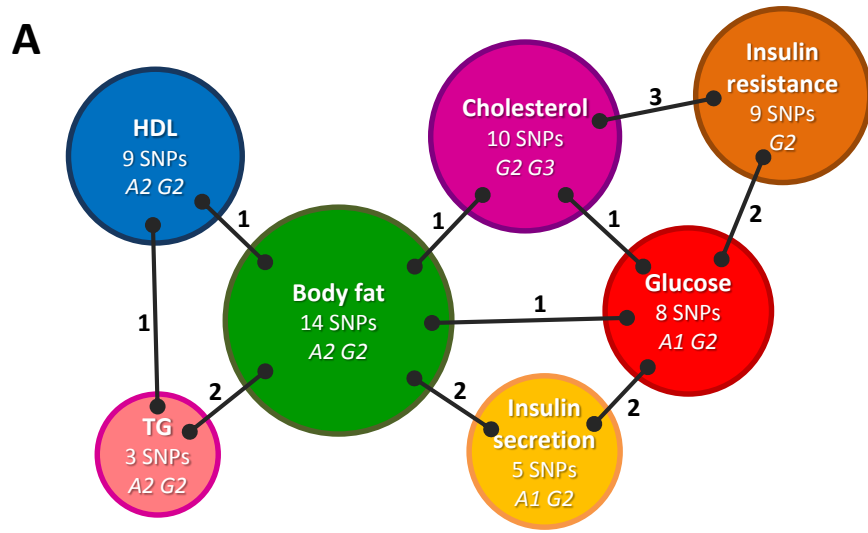
Metabolic trait	p ₁	p ₂	p ₃	effect size p.a. (adj.)
BMI	1.8E-07	2.3E-07	-	+0.22±0.05 kg/m ²
Body fat content	6.3E-05	1.5E-05	-	+0.18±0.05 %
Waist-hip ratio	0.0003	9.1E-07	-	+0.0018±0.0004
HOMA-IR	3.8E-07	4.7E-07	-	+0.06±0.02·10 ⁻⁶ mol·U/L ²
ISI	8.1E-06	9.9E-06	-	-0.12±0.04·10 ¹⁹ L ² /mol ²
C-peptide _{AUC0-30} /glucose _{AUC0-30}	0.2	0.2	0.2	-
Glucose ₀	0.0155	0.0149	0.8	+0.005±0.003 mmol/L
Glucose ₁₂₀	0.0035	0.0048	0.6	+0.02±0.01 mmol/L
Total cholesterol	0.8	0.9	-	-
LDL-cholesterol	0.6	0.6	-	-
HDL-cholesterol	0.2	0.1	-	-
Triglycerides	0.5	0.5	-	-

Score-phenotype associations were studied by linear regression analysis. Subscript numbers indicate time-points of oral glucose tolerance test. Adj., adjusted for gender and age; AMPK, AMP-activated protein kinase; AUC, area under the curve; BMI, body mass index; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index; LDL, low-density lipoprotein; p₁, p-value of unadjusted univariate analysis; p₂, p-value upon adjustment for gender and age; p₃, p-value upon adjustment for gender, age and ISI; p.a., per allele

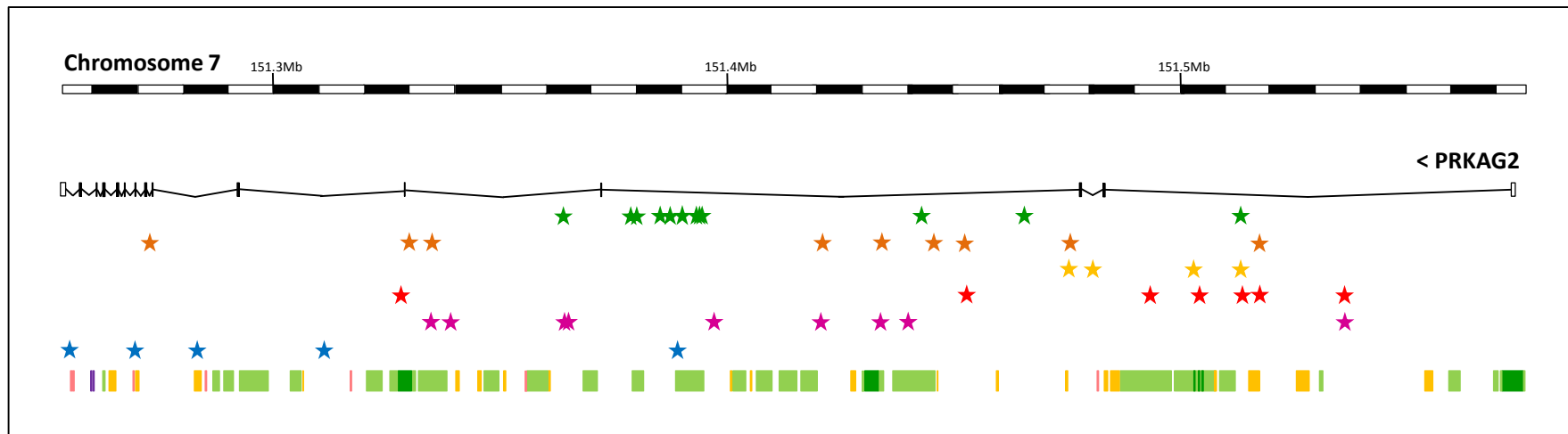
Table 3. Associations of the AMPK insulin resistance-, insulin secretion- and glucose scores with their respective metabolic traits and blood glucose levels

Metabolic trait	Insulin resistance score			Insulin secretion score			Glucose score		
	p ₁	p ₂	effect size p.a. (adj.)	p ₁	p ₂	effect size p.a. (adj.)	p ₁	p ₂	effect size p.a. (adj.)
HOMA-IR	2.4E-07	1.1E-06	+0.07±0.02·10 ⁻⁶ mol·U/L ²	-	-	-	-	-	-
ISI	2.3E-06	1.2E-05	-0.14±0.06·10 ¹⁹ L ² /mol ²	-	-	-	-	-	-
C-peptide _{AUC0-30} /glucose _{AUC0-30}	-	-	-	3.0E-06	3.4E-05	-3.2±0.9·10 ⁻⁹	-	-	-
Glucose ₀	1.9E-05	0.0001	+0.01±0.004 mmol/L	3.3E-05	0.0008	+0.01±0.007 mmol/L	9.5E-09	1.4E-07	+0.02±0.005 mmol/L
Glucose ₁₂₀	0.0118	0.07	-	0.0001	0.0008	+0.05±0.02 mmol/L	3.3E-08	2.2E-07	+0.07±0.02 mmol/L

Score-phenotype associations were studied by linear regression analysis. Subscript numbers indicate time-points of oral glucose tolerance test. Adj., adjusted for gender and age; AMPK, AMP-activated protein kinase; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index; p₁, p-value of unadjusted univariate analysis; p₂, p-value upon adjustment for gender and age; p.a., per allele



B



Regulatory sites
 ■ Promoter
 ■ Promoter flank
 ■ Enhancer
 ■ TF-binding site
 ■ Open chromatin

SNPs
 ★ Body fat SNPs
 ★ Insulin resistance SNPs
 ★ Insulin secretion SNPs
 ★ Glucose SNPs
 ★ Cholesterol SNPs
 ★ HDL SNPs

Fig. 1

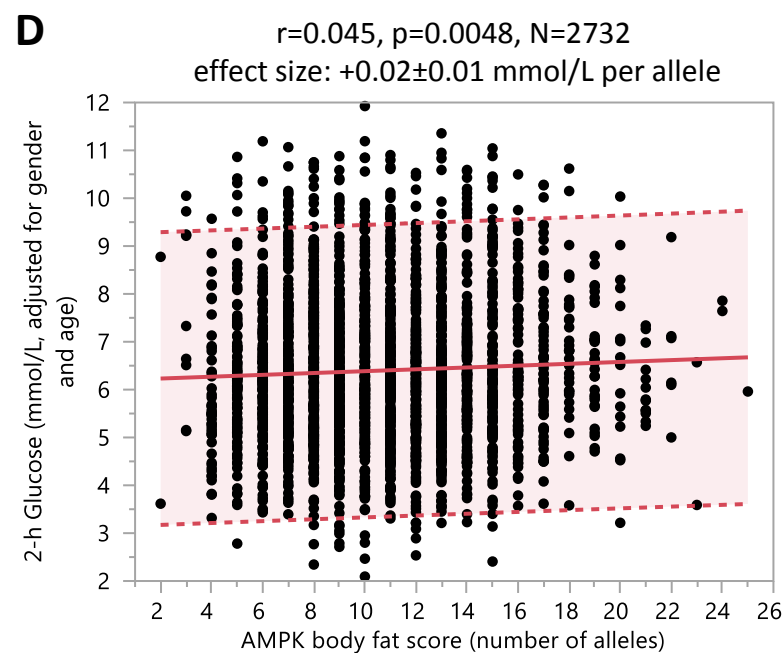
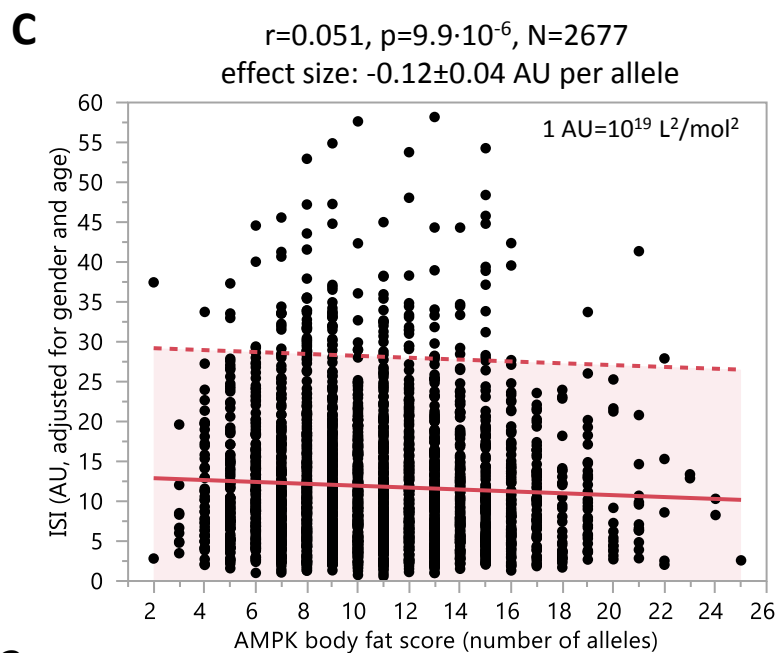
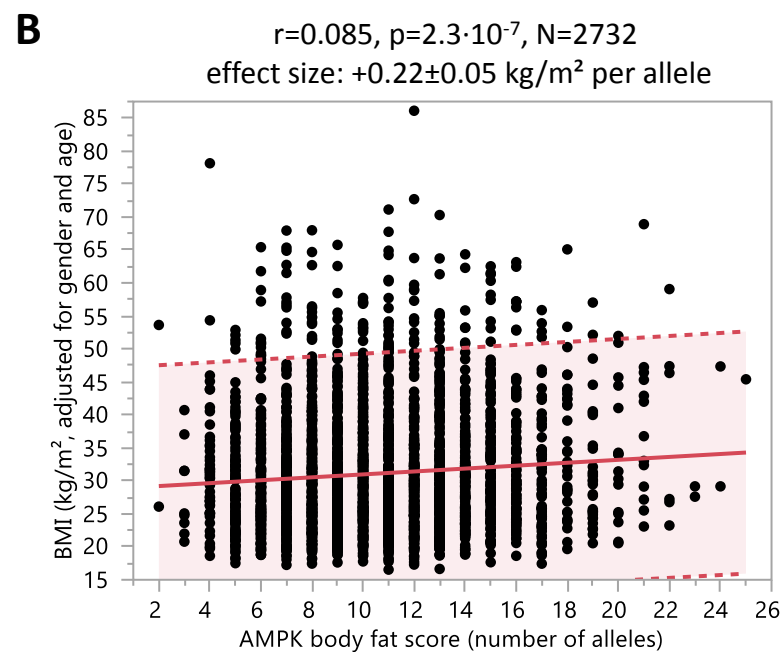
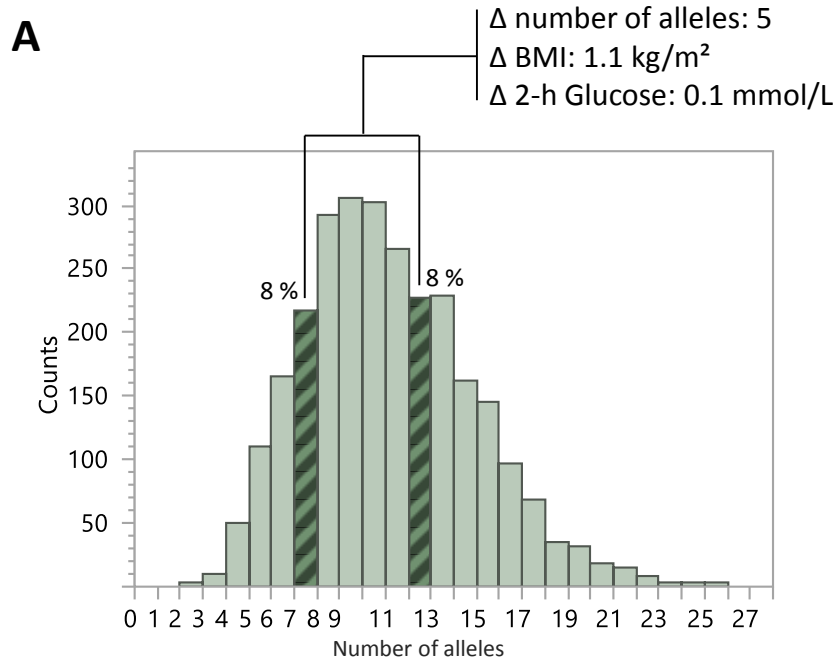


Fig. 2

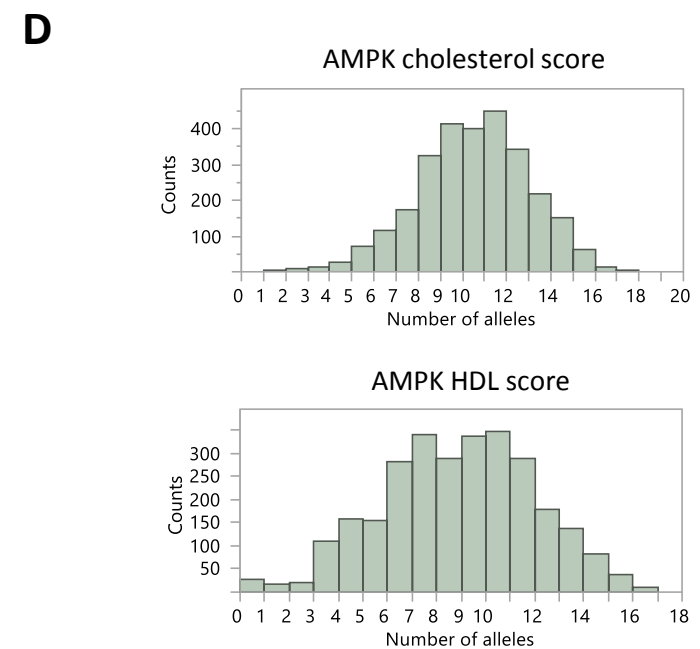
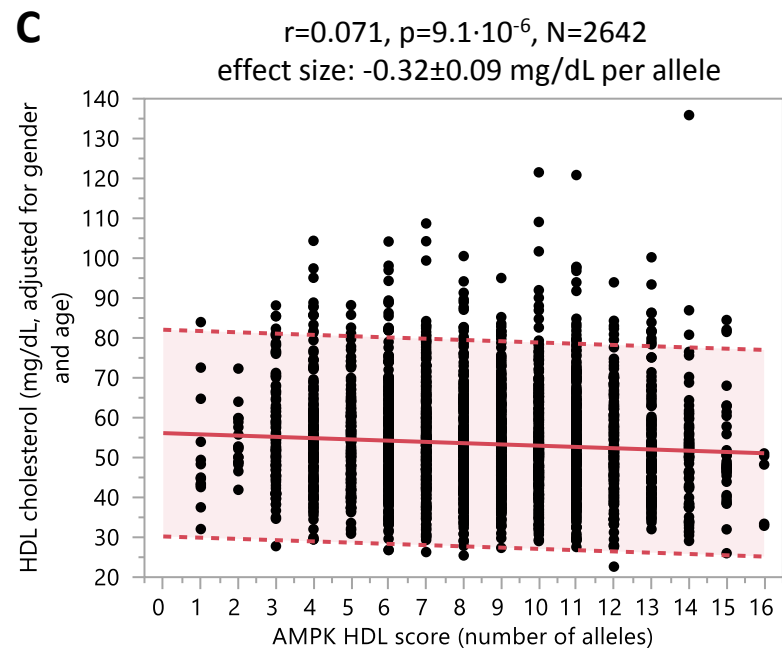
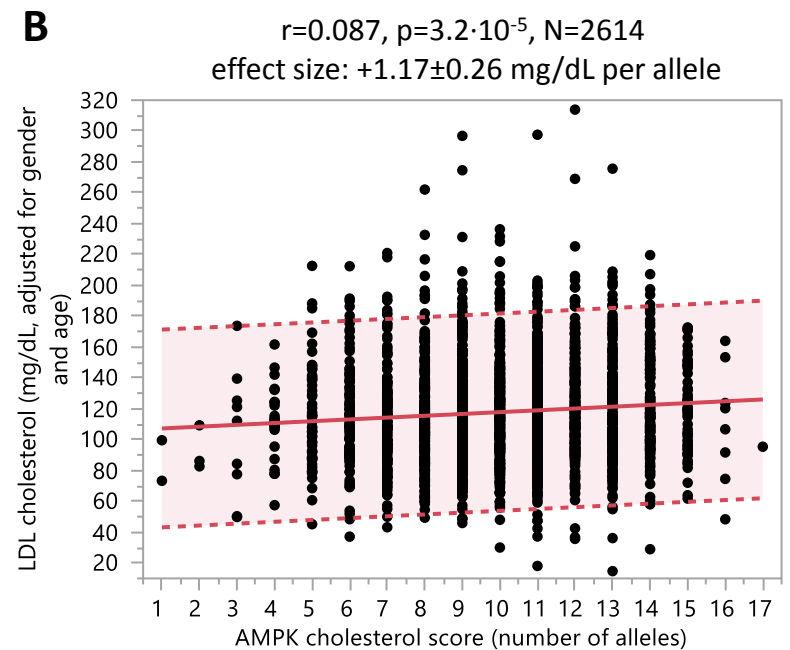
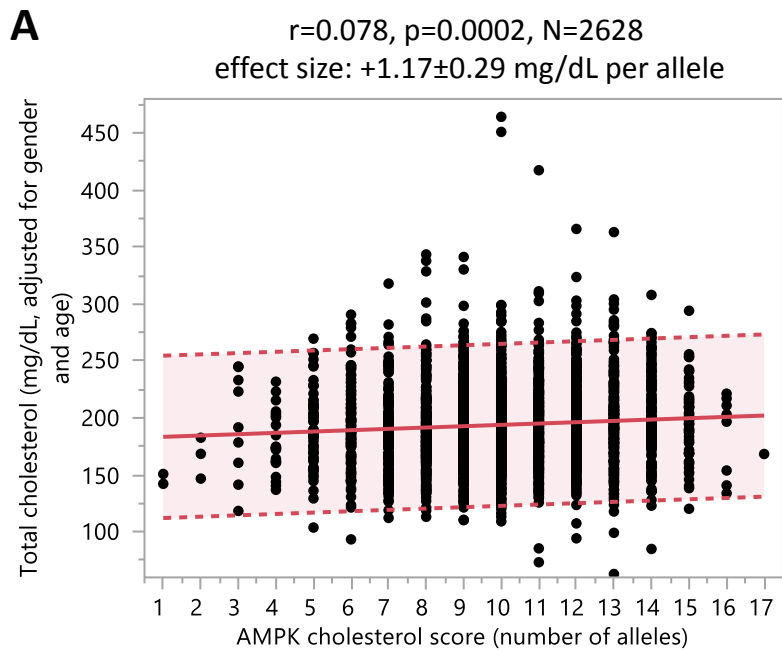


Fig. 3