Circulating Fetuin-A and Risk of Type 2 Diabetes: A Mendelian Randomization Analysis

Janine Kröger,[1,2] Karina Meidtner,[1,2] Norbert Stefan,[2,3,4] Marcela Guevara,[5,6,7] Nicola D. Kerrison,[8] Eva Ardanaz,[5,6,7] Dagfinn Aune,[9,10] Heiner Boeing,[11] Miren Dorronsoro,[7,12,13] Courtney Dow,[14,15,16] Guy Fagherazzi,[14,15,16] Paul W. Franks,[17,18] Heinz Freisling,[19] Marc J. Gunter,[19] José María Huerta,[7,20] Rudolf Kaaks,[21] Timothy J. Key,[22] Kay Tee Khaw,[23] Vittorio Krogh,[24] Tilman Kühn,[25] Francesca Romana Mancini,[14,15,16] Amalia Mattiello,[26] Peter M. Nilsson,[17] Anja Olsen,[27] Kim Overvad,[28,29] Domenico Palli,[30] J. Ramón Quirós,[31] Olov Rolandsson,[18] Carlotta Sacerdote,[32,33] Núria Sala,[34] Elena Salamanca-Fernández,[7,35,36] Ivonne Sluijs,[37] Annemieke M.W. Spijkerman,[38] Anne Tjonneland,[27] Konstantinos K. Tsilidis,[9,39] Rosario Tumino,[40,41] Yvonne T. van der Schouw,[37] Nita G. Forouhi,[8] Stephen J. Sharp,[8] Claudia Langenberg,[8] Elio Riboli,[42] Matthias B. Schulze,[1,2,43] and Nicholas J. Wareham[8]

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Fetuin-A, a hepatic-origin protein, is strongly positively associated with risk of type 2 diabetes in human observational studies, but it is unknown whether this association is causal. We aimed to study the potential causal relation of circulating fetuin-A to risk of type 2 diabetes in a Mendelian randomization study with single nucleotide polymorphisms located in the fetuin-A–encoding *AHSG* gene. We used data from eight European countries of the European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct case-cohort study including 10,020 incident cases. Plasma fetuin-A concentration was measured in a subset of 965 subcohort participants and 654 case subjects. A genetic score of the *AHSG* single nucleotide polymorphisms was strongly associated with fetuin-A (28% explained variation). Using the genetic score as instrumental variable of fetuin-A, we observed no significant association of a 50 μg/mL higher fetuin-A concentration with diabetes risk (hazard ratio 1.02 [95% CI 0.97, 1.07]). Combining our results with those from the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortium (12,171 case subjects) also did not suggest a clear significant relation of fetuin-A with diabetes risk. In conclusion, although there is mechanistic evidence for an effect of fetuin-A on insulin sensitivity and secretion, this study does not support a strong, relevant relationship between circulating fetuin-A and diabetes risk in the general population.

[1]Department of Molecular Epidemiology, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany
[2]German Center for Diabetes Research (DZD), München-Neuherberg, Germany
[3]Department of Internal Medicine IV, University Hospital Tübingen, Tübingen, Germany
[4]Institute for Diabetes Research and Metabolic Diseases, Helmholtz Centre München, University of Tübingen, Tübingen, Germany
[5]Navarre Public Health Institute, Pamplona, Spain
[6]Instituto de Investigación Sanitaria de Navarra (Navarra Institute for Health Research), Pamplona, Spain
[7]CIBER de Epidemiología y Salud Pública, Madrid, Spain
[8]MRC Epidemiology Unit, University of Cambridge, Cambridge, U.K.
[9]Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, London, U.K.
[10]Bjørknes University College, Oslo, Norway
[11]German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany
[12]Public Health Division of Gipuzkoa, San Sebastian, Spain
[13]Instituto BioDonostia, Basque Government, San Sebastian, Spain
[14]Center for Research in Epidemiology and Population Health, INSERM U1018, Villejuif, France
[15]Paris-Sud, Paris-Saclay University, Villejuif, France
[16]Gustave Roussy Institute, Villejuif, France
[17]Lund University, Malmö, Sweden
[18]Umeå University, Umeå, Sweden
[19]Section of Nutrition and Metabolism, International Agency for Research on Cancer, World Health Organization, Lyon, France
[20]Department of Epidemiology, Murcia Regional Health Council, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca, Murcia, Spain
[21]Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany
[22]University of Oxford, Oxford, U.K.
[23]University of Cambridge, Cambridge, U.K.
[24]Istituto Nazionale dei Tumori, Fondazione IRCCS, Milan, Italy
[25]German Cancer Research Center, Heidelberg, Germany
[26]Dipartimento di Medicina Clinica e Chirurgia, Federico II University, Naples, Italy
[27]Danish Cancer Society Research Center, Copenhagen, Denmark
[28]Section for Epidemiology, Department of Public Health, Aarhus University, Aarhus, Denmark
[29]Aalborg University Hospital, Aalborg, Denmark
[30]Cancer Research and Prevention Institute, Florence, Italy

Fetuin-A is a protein produced primarily by the liver, particularly in nonalcoholic fatty liver disease (1,2). Fetuin-A is an inhibitor of the insulin receptor at the tyrosine kinase level (3,4). Fetuin-A knockout mice were protected against insulin resistance (5,6), and strong direct associations between fetuin-A levels and diabetes risk have been observed in prospective observational studies (7–13).

Fetuin-A is encoded by the *AHSG* gene. Earlier studies have shown strong associations of *AHSG* single nucleotide polymorphisms (SNPs) with fetuin-A levels (14,15). The Mendelian randomization approach, which involves the use of genetic information as an instrumental variable of the exposure, can be applied to estimate the potential causal effect of fetuin-A on diabetes risk (16). So far, only one small study has applied the Mendelian randomization approach to investigate fetuin-A in relation to risk of type 2 diabetes (17). This study suggested no causal involvement of fetuin-A (17); however, it was limited by low power to detect associations.

We therefore used the data of the multicenter European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct study, with more than 10,000 incident cases of type 2 diabetes, to estimate the unconfounded effect of fetuin-A on diabetes risk with a Mendelian randomization approach. To maximize power, we additionally incorporated data from the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortium, which had 12,171 subjects with type 2 diabetes.

RESEARCH DESIGN AND METHODS

EPIC-InterAct study

The EPIC-InterAct study is a case-cohort study nested within the prospective EPIC study (18). In brief, EPIC includes 519,978 men and women, mostly aged 35–70 years, who were recruited between 1991 and 2000 at 23 centers in 10 European countries participating in EPIC (19). Each EPIC center obtained individual written informed consent and local ethics approval.

For the EPIC-InterAct study, incident cases of type 2 diabetes occurring in the EPIC cohort were ascertained and verified. All EPIC countries except Norway and Greece contributed to EPIC-InterAct (n = 455,680). Individuals without stored blood (n = 109,625) or without information on reported diabetes status (n = 5,821) were excluded, leaving 340,234 participants eligible for inclusion in EPIC-InterAct (18).

Case-Cohort Construction and Case Ascertainment

A center-stratified, random subcohort of 16,835 individuals was selected. After exclusion of 548 individuals with prevalent diabetes and 133 with uncertain diabetes status, the subcohort included 16,154 individuals for analysis (18).

Details about the ascertainment of cases can be found in the Supplementary Data. Altogether, 12,403 verified incident case subjects were identified (18). In total, the EPIC-InterAct study involves 27,779 participants (16,154 subcohort members, 12,403 incident case subjects, including 778 case subjects within the subcohort) (18).

Study Population for the Present Analysis

Figure 1 provides an overview of the study populations. As fetuin-A had been measured in Potsdam only, analyses involving fetuin-A were based on the data of 1,593 participants from Potsdam (965 subcohort members, 654 incident case subjects, including 26 case subjects within the subcohort). Instrumental variable analysis was performed in the whole of EPIC-InterAct, excluding participants whose genetic data were not usable (n = 5,287 due to insufficient amount of DNA, low call rate, X chromosome heterozygosity concordance with self-reported sex, outliers for heterozygosity, or lack of concordance with previous genotyping results) and EPIC-Potsdam participants with missing (n = 16) or implausible (n = 97) fetuin-A data, resulting in a data set of 22,379 men and women (12,975 subcohort members, 10,020 incident case subjects). Analyses of the association of SNPs with potential confounders or mediators were performed after further exclusion of individuals (n = 3,946) with missing values for these variables (data set of 18,433 participants with 10,850 subcohort members and 8,108 case subjects).

Measurement of Fetuin-A and Other Biomarkers

Blood samples were taken at baseline. For the Potsdam participants, plasma levels of fetuin-A were measured in Tübingen, Germany, with the automatic ADVIA 1650 analyzer (Siemens Medical Solutions, Erlangen, Germany). An immunoturbidimetric method was used with specific polyclonal goat

[31]Public Health Directorate, Asturias, Spain
[32]Unit of Cancer Epidemiology, Città della Salute e della Scienza Hospital–University of Torino, Center for Cancer Prevention, Torino, Italy
[33]Human Genetics Foundation, Torino, Italy
[34]Unit of Nutrition and Cancer, Cancer Epidemiology Research Programme, and Translational Research Laboratory, Catalan Institute of Oncology, Barcelona, Spain
[35]Andalusian School of Public Health, Granada, Spain
[36]Instituto de Investigación Biosanitaria de Granada, Granada, Spain
[37]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[38]National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[39]Department of Hygiene and Epidemiology, University of Ioannina School of Medicine, Ioannina, Greece
[40]Azienda Sanitaria Provinciale di Ragusa, Ragusa, Italy
[41]L'Associazione Iblea per la Ricerca Epidemiologica–Un'organizzazione Non Lucrativa di Utilità Sociale, Ragusa, Italy
[42]School of Public Health, Imperial College London, London, U.K.
[43]Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany
Corresponding author: Matthias B. Schulze, mschulze@dife.de.
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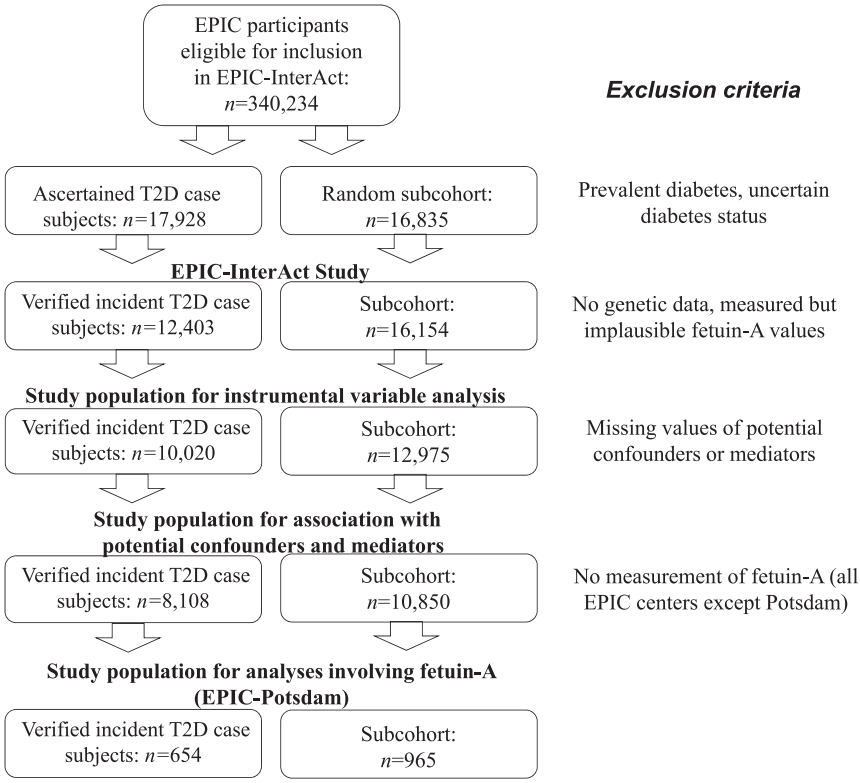

Figure 1—Construction of the EPIC-InterAct case-cohort study and the study populations used for the present analysis. T2D, type 2 diabetes.

anti-human fetuin-A antibodies to human fetuin-A (BioVendor Laboratory Medicine, Brno, Czech Republic). This method was evaluated in a side-by-side comparison with an ELISA (intra-assay coefficient of variation 3.5%, interassay coefficient of variation 5.4%; BioVendor), showing a correlation of $r = 0.93$.

All other laboratory measures were done for all EPIC-InterAct participants by the Stichting Huisartsen Laboratorium Groep (Etten-Leur, the Netherlands) (Supplementary Data).

Genotyping, Identification of Tagging SNPs, and Construction of Genetic Scores

Details about genotyping methods can be found in the Supplementary Data.

We selected five tagging SNPs of the *AHSG* gene (rs4917, rs2070635, rs2070633, rs2248690, rs4831) using HapMap 22/phase II Centre d'Etude du Polymorphism Humain population data applying stringent criteria (minor allele frequency >5%, pairwise $r^2 \geq 0.80$) as similarly done earlier (14). A genetic score was created by summing the number of fetuin-A–raising alleles of the single SNPs. We also constructed a weighted genetic score to account for the different strength of association between SNPs and fetuin-A (details of instrumental variables described in the Supplementary Data).

Assessment of Other Covariables

Standardized questionnaires were used at baseline to collect information on sociodemographic characteristics and lifestyle, including age, education level, smoking status, occupational and leisure-time physical activity, and history of previous illness. Height, weight, and waist circumference of participants were obtained by trained staff during the baseline examination using standardized protocols (20). However, for participants from France and some participants from Oxford (U.K.), self-reported anthropometric data were collected.

Statistical Analysis

The associations of SNPs with fetuin-A were determined by linear regression in the Potsdam subcohort with SNPs modeled per fetuin-A–increasing allele (additive model). First, the individual SNPs were analyzed in separate models. Then, all SNPs were included in the same model. SNPs showing a significant association with fetuin-A in this model were used to generate the genetic scores, with β-coefficients used as weights.

The associations of the genetic score with potential confounders or mediators were evaluated by linear regression for continuous and logistic regression for dichotomous confounders/mediators.

To study the association of genetic variables with diabetes, we performed Cox regression with Prentice weighting (21). Age was used as underlying time scale. Models were stratified by integers of age (years), study center, and genotyping source and further adjusted for sex. Heterogeneity between countries in the association of the genetic variables with diabetes risk was investigated by computing country-specific hazard ratios (HRs) and pooling these with random-effects meta-analyses.

We calculated instrumental variable estimates to estimate the unconfounded effect of an increase of 50 μg/mL in fetuin-A on diabetes risk. We assumed very similar associations of *AHSG* SNPs and fetuin-A concentration between the Potsdam center and the other EPIC-InterAct centers and applied the two-stage least squares procedure (22) for calculating instrumental variable estimates.

To increase power, we further included data from the DIAGRAM consortium (Supplementary Data). Summary statistics were meta-analyzed with the odds ratio (OR) and 95% CI of the respective SNP derived from EPIC-InterAct (after excluding the Norfolk [U.K.] center because this is included in DIAGRAM) using a fixed-effects model.

Statistical analyses were performed with SAS (version 9.4, Enterprise Guide 6.1; SAS Institute, Cary, NC).

Furthermore, we performed a power calculation using the online tool mRnd (http://cnsgenomics.com/shiny/mRnd/) (23), as described in the Supplementary Data.

RESULTS

Table 1 shows baseline characteristics for the subcohort members of the EPIC-InterAct study and of the Potsdam center.

Observational Analysis of Fetuin-A and Diabetes Risk

In EPIC-Potsdam, circulating fetuin-A was positively associated with diabetes, with a HR of 1.18 (95% CI 1.05, 1.33) per 50 μg/mL in the multivariable-adjusted model (data not shown). Additional adjustment for BMI and waist circumference did not alter the observed association.

Table 1—Baseline characteristics of subcohort participants of the EPIC-InterAct study (*n* = 10,850) and the Potsdam center (*n* = 965)

	EPIC-InterAct	Potsdam center
General characteristics		
Age, years	53 (9)	50 (9)
Male	37	39
BMI (kg/m^2)	26.1 (4.2)	25.7 (4.1)
Current smoking	27	19
Physically active	21	16
Longer education	21	38
Alcohol intake (g/day)	7.4 (1.2, 20.1)	8.2 (3.1, 19.5)
Biomarkers		
Fetuin-A, μg/mL	—	267 (61)
GGT, units/L	20 (14, 32)	21 (15, 35)
ALT, units/L	18 (14, 25)	19 (14, 27)
Albumin, g/L	46 (3)	46 (3)
Creatinine, μmol/L	68 (60, 78)	69 (61, 80)
CRP, mg/L	1.1 (0.6, 2.4)	1.1 (0.6, 2.3)
HDL cholesterol, mmol/L	1.5 (0.4)	1.5 (0.4)
Total cholesterol, mmol/L	6.0 (1.1)	5.9 (1.1)
Triglycerides, mmol/L	1.1 (0.8, 1.7)	1.1 (0.8, 1.7)
HbA_{1c}, % (mmol/mol)	5.4 [5.2, 5.7] (36 [33, 39])	5.4 [5.1, 5.5] (35 [32, 37])

Data are mean (SD), median (interquartile range), or %. ALT, alanine aminotransferase; CRP, C-reactive protein; GGT, γ-glutamyl transferase.

Association of *AHSG* SNPs and Genetic Scores With Fetuin-A and Diabetes Risk

In the Potsdam subcohort, four of the five *AHSG* SNPs were significantly associated with fetuin-A in univariate analyses (Supplementary Table 1). Including all five SNPs simultaneously in one model revealed significant associations for three SNPs (Table 2), which were subsequently used to create the genetic scores. Both the weighted (weighted by β-coefficients from linear regression) and the unweighted genetic score showed a strong association with fetuin-A, explaining 28% and 27% of variation in fetuin-A, respectively. Neither genetic score was associated with diabetes risk (Supplementary Figs. 1 and 2).We detected no meaningful association of the weighted genetic score with potential confounders or mediators in the EPIC-InterAct subcohort (Supplementary Tables 2 and 3).

Instrumental Variable Analysis of Fetuin-A and Diabetes

Using instrumental variable analysis to estimate the unconfounded association for a 50 μg/mL higher fetuin-A level did not indicate an effect on diabetes risk in EPIC-InterAct. The HR for the weighted genetic score was 1.02 (95% CI 0.97, 1.07). Also for single SNPs, the HRs were generally very low in magnitude (between 1.01 and 1.04) and nonsignificant (Table 3). When further combining EPIC-InterAct with DIAGRAM, the ORs for the single SNPs remained the same, but ORs for two SNPs became borderline significant (OR for rs4917 = 1.02 [95% CI 1.00, 1.06], OR for rs2248690 = 1.04 [95% CI 1.00, 1.08]).

We also analyzed potential differences in the association of the weighted genetic score with diabetes risk in EPIC-InterAct. Stratification by age, sex, waist

Table 2—Association of individual *AHSG* SNPs and genetic scores with fetuin-A concentration in the Potsdam part of the EPIC-InterAct study subcohort (*n* = 965): simultaneous adjustment of SNPs

	Allele	β (SE) for fetuin concentration	*P*	F statistic
Individual SNPs				
rs4917	T→C	16.9 (8.4)	0.0446	<0.0001
rs2070635	G→A	−0.6 (5.1)	0.9013	
rs2070633	T→C	23.7 (8.2)	0.0040	
rs2248690	T→A	12.3 (4.3)	0.0040	
rs4831	G→C	1.1 (8.6)	0.9008	
Genetic scores*				
Weighted score		52.4 (2.8)	<0.0001	<0.0001
Unweighted score		53.0 (2.8)	<0.0001	<0.0001

β for individual SNPs obtained from one linear regression model including all five SNPs simultaneously. β for genetic scores obtained from single univariate linear regression models. SNPs and genetic scores modeled per fetuin-A–increasing allele and fetuin-A expressed in μg/mL. *Genetic scores were created as sum of fetuin-A–increasing alleles of rs4917, rs2070633, and rs2248690, divided by three. β of the three SNPs from linear regression have been used as weights for the weighted genetic score.

Table 3—Instrumental variable estimates for the weighted genetic score and single *AHSG* SNPs reflecting the association of a 50 μg/mL increase in fetuin-A concentration with diabetes risk

	Weighted genetic score	rs4917	rs2070633	rs2248690
EPIC-InterAct*	1.02 (0.97, 1.07)	1.02 (0.98, 1.07)	1.01 (0.95, 1.08)	1.04 (0.96, 1.10)
EPIC-InterAct and DIAGRAM combined†		1.02 (1.00, 1.06)	1.01 (0.98, 1.05)	1.04 (1.00, 1.08)

Data are HRs (for EPIC-InterAct) or ORs (for EPIC-InterAct and DIAGRAM combined) and 95% CIs per 50 μg/mL increase in fetuin-A concentration. Instrumental variable estimates were obtained from two-stage least squares procedure. Estimates for EPIC-InterAct and DIAGRAM have been combined with a fixed-effects meta-analysis. *EPIC-InterAct: n = 22,379 including 12,975 incident case subjects with type 2 diabetes. †DIAGRAM: n = 69,033 including 12,171 incident case subjects with type 2 diabetes.

circumference, or HbA_{1c} did not reveal meaningful differences between subgroups (Supplementary Table 4).

DISCUSSION

In this large European case-cohort study, instrumental variables reflecting circulating fetuin-A were not significantly associated with diabetes risk. Further meta-analyzing results with those from the DIAGRAM consortium to bolster statistical power also did not indicate a strong association of genetically elevated fetuin-A with diabetes.

Several prospective observational studies have detected a significant and strong positive association between plasma fetuin-A levels and the risk of type 2 diabetes (7–13). We could not confirm this strong association in a Mendelian randomization approach. Although we found borderline significant estimates for two SNPs (OR for rs4917 = 1.02 [95% CI 1.00, 1.06], OR for rs2248690 = 1.04 [95% CI 1.00, 1.08]), our results do not suggest a strong causal association because an allele increase corresponds to a high difference in fetuin-A (50 μg/mL, SD for fetuin-A = 61 μg/mL). According to our results of the observational analysis, we would have expected an OR of 1.18. Due to the use of different assays to determine concentrations of fetuin-A across studies, we could not calculate expected ORs based on the other observational studies on fetuin-A and diabetes risk. The slightly stronger associations of *AHSG* SNPs with fetuin-A (17) than in EPIC-Potsdam and the results of quantile comparisons (7–9,11,12) suggest even higher expected ORs based on these studies.

Our study was not designed to unravel specific factors that explain the results of earlier observational studies, but it suggests that fetuin-A concentration more strongly reflects an adverse metabolic profile than an important causal risk factor in the pathogenesis of diabetes.

Our results are in agreement with those from an earlier small study on *AHSG* SNPs and diabetes risk involving older adults (17). Fetuin-A was positively associated with fasting glucose levels, but *AHSG* SNPs did not support an association between genetically predicted fetuin-A concentrations and fasting glucose or diabetes risk. A major limitation of this study is the small study size, resulting in a low power for the instrumental variable analysis (3,435 participants including 259 incident case subjects).

In experiments in mice and human adipocytes, it has been observed that free fatty acids (FFAs) require the presence of fetuin-A to induce an inflammatory signaling pathway resulting in insulin resistance and subclinical inflammation (24). Fetuin-A might indeed contribute to the development of type 2 diabetes in already predisposed individuals with high concentrations of FFAs. Supporting this assumption, an interaction of fetuin-A and FFAs in determining insulin sensitivity was observed in a study of 347 participants at increased risk for type 2 diabetes and cardiovascular disease (25). In our study, the unavailability of FFA blood concentrations precluded us from investigating a potential effect modification by FFAs.

A major strength of our study relates to the large sample size for analyzing *AHSG* SNPs and genetic scores in relation to diabetes risk. *AHSG* SNPs and genetic scores showed strong associations with circulating fetuin-A resulting in powerful instrumental variables. All SNPs are located in the fetuin-A–encoding *AHSG* gene, making pleiotropic roles of the SNPs rather unlikely. A limitation of our study is that the information on circulating fetuin-A was only available in the Potsdam center of EPIC-InterAct.

In conclusion, although there is mechanistic evidence for an effect of fetuin-A on insulin sensitivity and secretion, this study does not support a strong, relevant relationship between circulating fetuin-A and diabetes risk in the general population.

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