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Association Studies Article

ASSOCIATION STUDIES ARTICLE

Detection of genetic loci associated with plasma fetuin-A: a meta-analysis of genome-wide association studies from the CHARGE Consortium

Majken K. Jensen[1], Richard A. Jensen[2], Kenneth J. Mukamal[3], Xiuqing Guo[4], Jie Yao[4], Qi Sun[1], Marilyn Cornelis[5], Yongmei Liu[6], Ming-Huei Chen[7,20], Jorge R. Kizer[8], Luc Djoussé[9], David S. Siscovick[10], Bruce M. Psaty[2,11], Joseph M. Zmuda[12], Jerome I. Rotter[4], Melissa Garcia[13], Tamara Harris[14], Ida Chen[4], Mark O. Goodarzi[15], Michael A. Nalls[13], Margaux Keller[13], Alice M. Arnold[16], Anne B. Newman[12], Ron C. Hoogeveen[17], Kathryn M. Rexrode[18], Eric B. Rimm[1], Frank B. Hu[1], Vasan S. Ramachandran[19,20], Ronit Katz[4], James S. Pankow[21] and Joachim H. Ix[22,*]

[1]Department of Nutrition, Harvard T. H. Chan School of Public Health, Boston, MA 02115, USA & Channing Division of Network Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA, [2]Cardiovascular Health Research Unit, Departments of Medicine, Epidemiology, and Health Services, University of Washington, Seattle, WA 98101, USA, [3]Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA, [4]Institute for Translational Genomics and Population Sciences, Los Angeles BioMedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA 90502, USA, [5]Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA, [6]Wake Forest University Health Sciences, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA, [7]Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA, [8]Department of Medicine & Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY 10461, USA, [9]Division of Aging, Brigham and Women's Hospital, Boston, MA 02115, USA, & Boston Veterans Affairs Healthcare System, Boston, MA 02130, USA, [10]New York Academy of Medicine, New York, NY, USA, [11]Group Health Research Institute and Group Health Cooperative, Seattle, WA 98101, USA, [12]Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA 15261, USA, [13]Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA, [14]Laboratory of Epidemiology and Populations Sciences, National Institute on Aging, Bethesda, MD 20892, USA, [15]Division of Endocrinology, Diabetes & Metabolism, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA, [16]Department of Biostatistics, University of Washington School of Public Health and Community Medicine, Seattle, WA 98195, USA, [17]Division of Atherosclerosis & Vascular Medicine, Baylor College of Medicine, Houston, TX 77030, USA, [18]Department of Medicine, Brigham and Women's Hospital, Boston, MA

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02115, USA, [19]Sections of Preventive Medicine and Epidemiology, and Cardiology & Department of Medicine, Boston University School of Medicine, Boston, MA 02118, Department of Epidemiology, Boston University School of Public Health, Boston, MA 02118, USA, [20]The Boston University and the National Heart, Lung and Blood Institute's Framingham Heart Study, Framingham, MA 01702, USA, [21]Division of Epidemiology and Community Health, University of Minnesota School of Public Health, Minneapolis, MN 55455, USA and [22]Nephrology Section, Veterans Affairs San Diego Healthcare System, University of California San Diego, San Diego, CA 92161, USA & Divisions of Nephrology and Preventive Medicine, Department of Medicine, University of California San Diego School of Medicine, San Diego, CA 92161, USA

*To whom correspondence should be addressed at: Division of Nephrology and Hypertension, Department of Medicine, University of California San Diego, and San Diego VA Healthcare System, 3350 La Jolla Village Drive. Mail code 111-H, San Diego, CA 92161, USA. Tel: 858 5528585, ext. 1657; Fax: 858 5527549; Email: joeix@ucsd.edu

Abstract

Plasma fetuin-A is associated with type 2 diabetes, and *AHSG*, the gene encoding fetuin-A, has been identified as a susceptibility locus for diabetes and metabolic syndrome. Thus far, unbiased investigations of the genetic determinants of plasma fetuin-A concentrations have not been conducted. We searched for single nucleotide polymorphisms (SNPs) related to fetuin-A concentrations by a genome-wide association study in six population-based studies.

We examined the association of fetuin-A levels with ~ 2.5 million genotyped and imputed SNPs in 9,055 participants of European descent and 2,119 African Americans. In both ethnicities, the strongest associations were centered in a region with a high degree of LD near the *AHSG* locus. Among 136 genome-wide significant ($P < 0.05 \times 10^{-8}$) SNPs near the *AHSG* locus, the top SNP was rs4917 ($P = 1.27 \times 10^{-303}$), a known coding SNP in exon 6 that is associated with a 0.06 g/l (~13%) lower fetuin-A level. This variant alone explained 14% of the variation in fetuin-A levels. Analyses conditioned on rs4917 indicated that the strong association with the *AHSG* locus stems from additional independent associations of multiple variants among European Americans. In conclusion, levels of fetuin-A in plasma are strongly associated with SNPs in its encoding gene, *AHSG*, but not elsewhere in the genome. Given the strength of the associations observed for multiple independent SNPs, the *AHSG* gene is an example of a candidate locus suitable for additional investigations including fine mapping to elucidate the biological basis of the findings and further functional experiments to clarify *AHSG* as a potential therapeutic target.

Introduction

Fetuin-A is a liver-derived protein that is involved in the metabolism of calcified minerals and the regulation of the insulin signaling (1). Fetuin-A forms complexes with circulating calcium and phosphorus and increases the solubility of these minerals (2), thereby inhibiting arterial calcium deposition. Fetuin-A also directly binds and inhibits the insulin receptor, resulting in insulin resistance (3–5). The fetuin-A knock-out mouse has improved insulin sensitivity by euglycemic clamp experiments, lower triglyceride and free fatty acid levels, resistance to weight gain, and less adiposity (6,7). High fetuin-A levels are associated with insulin resistance (8–10), interacts with circulating free fatty acids in determining insulin sensitivity and predicts incident diabetes (11–16). Thus, fetuin-A represents an emerging biomarker for improved diabetes risk assessment in clinical practice and a potential therapeutic target for primary or secondary prevention of type 2 diabetes.

The alpha-2-HS-glycoprotein (*AHSG*) locus on chromosome 3 (3q27) encodes the fetuin-A gene. Linkage studies have identified this region as a susceptibility locus for the metabolic syndrome and T2DM (17,18), and small case-control studies have reported strong relations between several single nucleotide polymorphisms (SNPs) identified from direct exon sequencing studies and plasma concentrations of fetuin-A (19–22). However, a comprehensive investigation of genetic determinants of fetuin-A levels has not been conducted, and it remains unknown if genetic loci distinct from the *AHSG* locus can be identified that regulate fetuin-A concentrations. Hence, to better understand the genetic control of fetuin-A levels we conducted a genome-wide association (GWA) analysis in six population-based studies, as part of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium.

Results

Our analyses included data from six studies of European Americans totaling 9,055 individuals and four studies comprising a total of 2,119 African Americans. The majority of the participants were female and the average age ranged from 52 years in ARIC to 75 years in CHS. Mean fetuin-A levels ranged from 0.43 ± 0.09 g/l in African Americans from CHS to 1.00 ± 0.41 g/l in European Americans in HABC. However, most studies had smaller standard deviations than HABC and mean fetuin-A levels averaged around 0.50 g/l (Table 1).

Separate meta-analyses of 2.5 million SNPs for European and African American participants who contributed to the study-specific genome-wide association analyses of fetuin-A levels, identified a very strong signal on chromosome 3q27 (Fig. 1, Supplementary Material, Fig. S1). In total, 136 SNPs at the *AHSG* locus achieved genome-wide significance among European Americans. The top SNP, rs4917 ($P\text{-value} = 1.27 \times 10^{-303}$), is located in the *AHSG* gene which encodes the fetuin-A protein. The genetic region and the linkage disequilibrium (LD) of SNPs with rs4917 according to r^2 in CEU are displayed in Supplementary Material, Figure S2A and B. The triangles represent the top two non-synonymous SNPs (rs4917 in purple

Table 1. Descriptive characteristics of the cohorts included in the genome-wide analysis according to ancestry

Cohort	N	Age, years	Women, %	Fetuin-A level, g/l	Prevalent diabetes, %
CHS/European	2824	74.9 (5.0)	60.6	0.48 (0.10)	15.7
CHS/African	727	73.2 (5.6)	63.4	0.43 (0.09)	20.9
ARIC/European	485	53.4 (5.6)	55.7	0.51 (0.07)	0
ARIC/African	366	51.9 (5.4)	55.7	0.47 (0.09)	0
HABC/European	403	73.9 (2.9)	56.8	1.00 (0.41)	13.9
HABC/African	350	73.5 (3.0)	54.9	0.91 (0.37)	20.6
MESA/European	1093	62.7 (10.1)	52.0	0.49 (0.11)	3.9
MESA/African	678	62.0 (10.1)	52.3	0.45 (0.10)	10.5
NHS/European	1029	59.9 (6.4)	100	0.46 (0.11)	9.3
FHS/European	3592	40.0 (8.7)	53.6	0.45 (0.18)	2.5

Numbers in table are Mean (SD) or percentage. ARIC= Atherosclerosis Risk in Communities Study; CHS = Cardiovascular Health Study; FHS = Framingham Heart Study; HABC= Health, Aging, and Body Composition (Health ABC) Study; MESA= Multi-Ethnic Study of Atherosclerosis, NHS= Nurses' Health Study.
Diabetes was defined as fasting blood glucose >125 mg/dL, a random blood glucose of >200 mg/dL, or use of insulin or oral hypoglycemic agents.

Table 2. Association of the top SNPs (rs4917 in European Americans; rs1900618 in African Americans) on chromosome 3 with fetuin-A levels in European and African Americans

	rs4917							
	European Americans (MAF: 0.32)				African Americans (MAF: 0.26)			
Cohort	N	β	SE	p	N	β	SE	P
CHS	2742	−0.0632	0.0023	2.47E-163	725	−0.0447	0.0046	3.58E-22
ARIC	485	−0.0546	0.0046	1.03E-32	366	−0.0302	0.0082	2.48E-04
HABC	403	−0.11	0.031	4.44E-04	350	0.001	0.029	0.98
MESA	NA	NA	NA	NA	678	−0.0433	0.0061	1.13E-12
NHS	741	−0.069	0.053	6.71E-50	NA	NA	NA	NA
FHS	3592	−0.080	0.0032	3.40E-80	NA	NA	NA	NA
Combined	7963	−0.0657	0.0018	1.27E-303	2119	−0.0413	0.0034	1.20E-34
	rs1900618							
	European Americans (MAF: 0.33)				African Americans (MAF: 0.33)			
Cohort	**N**	**β**	**SE**	**p**	**N**	**β**	**SE**	**p**
CHS	2742	−0.0635	0.0023	2.27E-162	725	−0.0505	0.0040	2.58E-36
ARIC	485	−0.0542	0.0046	2.10E-32	366	−0.0345	0.0074	3.02E-06
HABC	403	0.11	0.031	4.44E-04	350	−0.015	0.027	0.59
MESA	NA	NA	NA	NA	678	−0.0514	0.0057	3.35E-19
NHS	741	−0.0691	0.0053	6.57E-50	NA	NA	NA	NA
FHS	3592	−0.0805	0.0042	1.03E-80	NA	NA	NA	NA
Combined	7963	−0.0659	0.0018	6.44E-303	2119	−0.0477	0.003	1.58E-56

The rs4917 and rs1900618 were identified as the top SNPs among European Americans and African Americans, respectively. The SNPs are in almost perfect LD in HapMap CEU population (Caucasians): $D^{|}=1$ and $r^2=0.96$. In HapMap YRI population= $D^{|}=1$ and $r^2=0.57$.
NA = SNP not available.

and rs4918 in red). Detailed results on the 136 SNPs are available in the online Supplementary Material, Table S3. Though the list of genes in closest proximity to the SNPs includes *FETUB*, *CRYGS*, *DNAJB11*, *KGN1*, and *TBCCD1*, these genes are all within close distance to *AHSG*, as shown in Supplementary Material, Figure S2A. Each additional copy of the rs4917 minor allele (T variant allele, minor allele frequency =0.32) was associated with approximately 0.066 ±0.002 g/l lower fetuin-A level in each of the cohorts (Table 2). rs4917 alone explained 14% of the variation in fetuin-A levels among European Americans.

The plots in Supplementary Material, Figure S2 show that the strongest associations are centered in a region with a high degree of LD. However, as shown in Supplementary Material, Figure S2B, five of the SNPs with extremely low *P*-values ($<5\times10^{-235}$) were not in strong LD with rs4917/rs4918 (light blue shaded dots, Supplementary Material, Fig. S2B). These five SNPs (rs13098866, rs13080283, rs2077119, rs1029353, and rs2070635) were instead in tight LD with each other ($r^2<0.6$). Further, when we repeated the genome-wide association analyses conditioning on rs4917 to uncover any additional, independently associated SNPs, 34 SNPs at the *AHSG* locus were associated with fetuin-A concentrations ($P<5\times10^{-8}$). The five SNPs from Supplementary Material, Figure S2B were among the SNPs that remained strongly statistically significantly associated with fetuin-A levels in this analysis (all $P<5\times10^{-28}$ in the conditional analysis). Among the 34 SNPs, ten SNPs were new hits ($P<5\times10^{-8}$ in the conditional analysis only). The 10 SNPs were not in LD with rs4917, indicating that the strong association

Table 3. Additional SNPs that were statistically significantly associated with fetuin-A levels in European Americans only in the genome-wide analysis conditioned on rs4917

Association results from meta-analyses conditioned on rs4917								Prior association results (unconditional analysis)		
SNP/coded allele	**Position** (chr 3)	MAF	β	SE	P	**Closest Gene***	**Distance**ϒ	β	SE	P
rs6787344/c	187822535	0.15	−0.0277	0.0023	3.82E-32	AHSG	733	0.000	0.003	0.89
rs4831/c	187813663	0.84	0.0259	0.0024	2.92E-28	AHSG	120	−0.003	0.003	0.23
rs4686432/t	187800551	0.15	−0.0246	0.0023	3.70E-27	AHSG	12992	0.002	0.003	0.48
rs9842063/a	187804207	0.89	0.0287	0.0028	4.13E-25	AHSG	9336	0.001	0.003	0.71
rs9873987/t	187802726	0.09	−0.0323	0.0032	2.00E-23	AHSG	10817	−0.002	0.004	0.58
rs9872086/t	187828594	0.79	0.0175	0.0026	1.13E-11	AHSG	6792	−0.002	0.003	0.46
rs6444146/t	187794361	0.91	0.0177	0.0028	1.79E-10	DNAJB11	8079	−0.003	0.003	0.30
rs9841006/t	187775829	0.09	−0.0172	0.0028	5.42E-10	DNAJB11	4669	0.003	0.003	0.31
rs13317898/t	187740514	0.93	0.0216	0.0035	9.94E-10	CRYGS	1588	−0.002	0.004	0.62
rs12330837/a	187768400	0.08	−0.0191	0.0033	6.98E-09	TBCCD1	594	0.009	0.004	0.03

*Closest gene: shows the gene-encoding region most closely situated to the given SNP.

ϒDistance: is the distance in base pairs from the closest known gene.

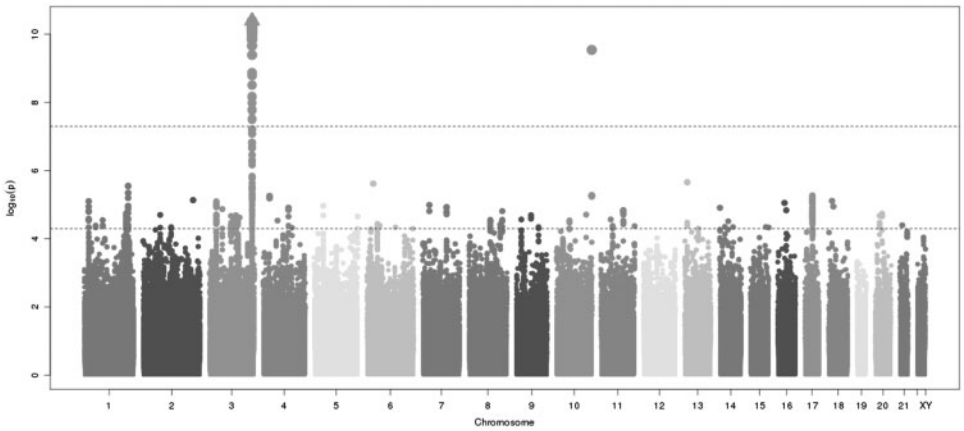

Figure 1. Meta-analysis of six genome-wide association analyses of fetuin-A levels in a total of 9,055 European Americans. Figure displays p-value for association for each SNP on a log10 scale

with fetuin-A levels observed for the *AHSG* locus stems from more than a single hit (Data for the 10 SNPs shown in Table 3). The Manhattan plot for European Americans also shows that other associations on chromosome 3 were elevated, though still not statistically significant upon adjustment for rs4917 (Supplementary Material, Fig. S5).

The *AHSG* locus also had the strongest association in the meta-analysis of four genome-wide studies that contributed data from 2,119 African American participants (Fig. 2, Supplementary Material, Fig. S3). In total, 42 SNPs at the *AHSG* locus were associated with fetuin-A levels with p values $< 5\times10^{-8}$ among the African Americans (Supplementary Material, Fig. S4 and Table S4). Each copy of the rs4917 minor allele was associated with 0.041 ± 0.003 g/l lower fetuin-A concentration (P-value $= 1.20\times10^{-34}$) and this variant alone explained 13% of the variation in fetuin-A levels, but another *AHSG* variant (rs1900618) was the top SNP in the genome-wide analysis of the African American samples. rs4917 and rs1900618 are in complete LD according to D' from HapMap YRI population, but the two SNPs were only moderately correlated (r^2=0.57) and had slightly different MAFs in African American participants only (0.26 versus 0.33). In contrast, both D' and r^2 were nearly 1 in HapMap CEU. Overall, Supplementary Material, Figure S4 shows the extent of LD was less pronounced among African American participants. Of note the five variants that were in tight LD with each other but not with rs4917/rs4918 were also among the genome-wide significant SNPs in African Americans (Supplementary Material, Table S4). Because of the modest correlation of rs4917 with just a handful of the 42 fetuin-A associated SNPs, the SNPs that were significant in the conditional analyses (conditioning on rs4917) mostly overlapped with the significant SNPs in the unconditional analyses (Supplementary Material, Fig. S6). Two exceptions were rs1486336 and rs958629 that reached significance only in the conditional analysis (and had borderline p values in the unconditional analysis ($P \sim 1\times10^{-7}$).

Discussion

Very little is known about the genetic determination of circulating fetuin-A levels, a marker of diabetes and the metabolic syndrome. To our knowledge, the present study represents the first unbiased genome-wide search for genetic variants associated with fetuin-A concentrations. In the present paper, all of the major genetic determinants of fetuin-A levels were located in the fetuin-A encoding gene; *AHSG*. We further provide evidence that the genetic variation of importance to fetuin-A

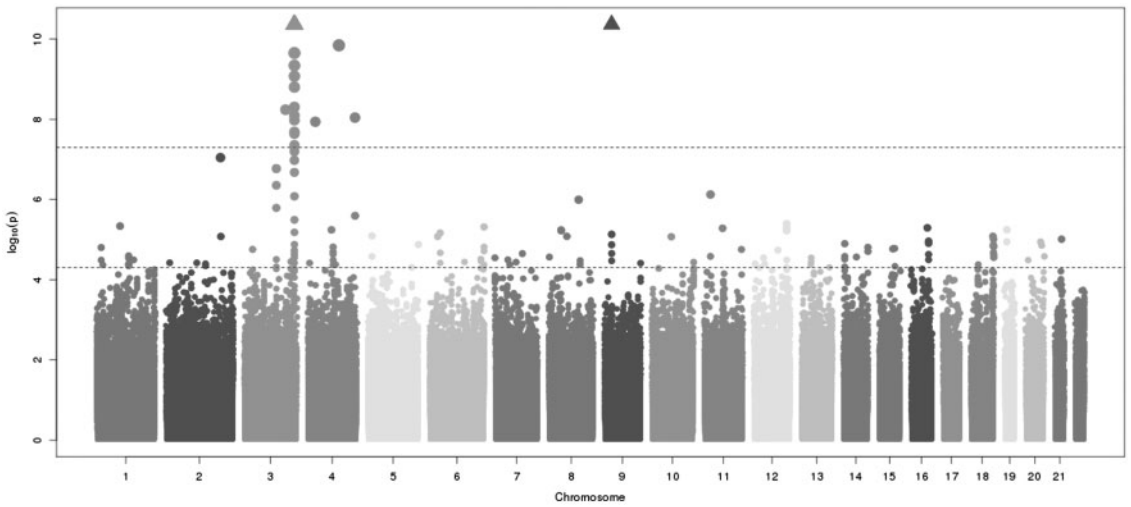

Figure 2. Meta-analysis of six genome-wide association analyses of fetuin-A levels in a total of 2,119 African Americans. Figure displays p-value for association for each SNP on a log10 scale

levels co-localize to the *AHSG* locus in both European and African Americans.

In both ethnicities, variants in and near the *AHSG* locus showed the strongest associations with fetuin-A levels. The most highly associated SNPs in the two ethnicities were not identical (rs4917 in European and rs1900618 in African-Americans), but showed remarkably similar associations that were in close proximity (within 1 kb distance) and were in very high linkage disequilibrium, suggesting that they may mark the same causal variant. Though the top association was different (rs1900618) among African American participants, the high degree of LD in this region does not allow for conclusions on the potential causal variant marked by these SNPs. Interestingly, genetic variation in *AHSG* has not been examined in African American samples before.

We found that rs4917 alone explained 14% of the variation in fetuin-A in European and 13% in African Americans individuals. In comparison, genome-wide association studies have identified several genetic loci that contribute to the levels of other biomarkers such as fibrinogen, CRP or adiponectin. Multigenetic risk scores that include all the independent genome-wide significant loci for each of these traits (e.g. up to 23 independent loci for fibrinogen) have been found to explain $< 4\%$ of the variation in fibrinogen and up to 5% for CRP and adiponectin (23–25).

When placed in the context of previous studies, several of the over 100 variants with genome-wide significant associations in our analysis have been identified previously. In particular, rs4917 and rs4918 are both missense variants located in the last two exons of the *AHSG* gene (exons 6 and 7, respectively) (26–28). Both SNPs have been associated with a similar magnitude of difference in plasma fetuin-A as observed in our study (0.06 ± 0.002 g/l lower levels in variant carriers) (22). While rs4917 and rs4918 do not appear to be related to metabolic markers including insulin levels, lipids, BMI, and fasting glucose in other studies (20,28–30) associations with risk of cardiovascular disease have been reported for both general (22,31) and renal patient populations (32).

AHSG expression is controlled by a number of transcriptional factors (TFs) such as C/EBP-[beta], NF-1, HNF-3[beta], AP-1 and ER[alpha] (33–36). The promoter SNP rs2248690, which has also been strongly associated with fetuin-A levels (26,29), modifies *AHSG* transcription by altering the affinity of AP-1 (26). Though this variant is located in the opposite region of *AHSG*, it is in LD with the exonic rs4917 and rs4918 SNPs ($r^2 > 0.80$ CEU), that potentially alter the DNA binding of several TFs including AP-1 and ER[alpha] (37). A transcriptional complex comprising ERα and AP-1 may contribute to estrogen-induced transcription of *AHSG* (36). A potential regulatory function stemming from the coding regions harboring rs4917 and rs4918 thus warrants further study.

In genome-wide analysis conditioning on rs4917, we identified 34 SNPs that were genome-wide significant in European Americans. Five SNPs were also significant in the main analysis (rs13098866, rs13080283, rs2077119, rs1029353, and rs2070635) because they were not in LD with rs4917, but in high LD with each other. Among these, rs2077119 in the 5′ untranslated region has previously been found to be associated with markers of insulin resistance in adipocytes (28), and with the risk of diabetes (20). A borderline association with risk of CHD has been reported for the intron variant rs2070635 (22). Another ten SNPs were only significantly associated with plasma fetuin-A levels in the analysis conditioned on rs4917. These 10 SNPs were in LD with rs2077119 ($D' > 0.7$ in HapMap CEU).

In conclusion, genetic variation in *AHSG* is strongly associated with fetuin-A levels. While we cannot exclude the possibility that other genetic loci play a role in fetuin-A concentrations, our sample size of over 9,000 European and 2,000 African Americans identified SNPs in the *AHSG* with *P*-values for their association lower than 1×10^{-300} indicating a very low chance that this is a false positive finding and highlighting the power inherent in analyses of this trait. Further work is needed to identify the functional variants and to understand the underlying biology of these associations. Our finding of a strong link between *AHSG* SNPs and fetuin-A levels provide a useful framework for the continued investigation of the causal variants in the *AHSG* gene.

Materials and Methods

We used data on genetic variation and fetuin-A levels from six population-based studies from the U.S. The following studies from the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium were included: Atherosclerosis Risk in Communities Study (ARIC), the Cardiovascular Health Study (CHS), the Multi-Ethnic Study of Atherosclerosis (MESA), and the Framingham Heart Study. In addition, investigators from the Health, Aging, and Body Composition (Health ABC) and Nurses' Health Study contributed data. Local ethical committees at each institution approved the individual study protocols. Detailed methods of each study population are included in the online supplement. Various genotyping platforms were used by the different studies, however, all studies imputed their genotyped data to HapMap release 22 CEU and YRI reference panels. Details on genotyping platform, quality control and imputation specifics are available in online in Supplementary Materials, Tables S1 and S2.

Fetuin-A measures

Fetuin-A was measured in plasma using enzyme linked immunosorbent assays (ELISA) from Epitope Diagnostics, San Diego, CA (CHS, MESA, HABC, and ARIC), Biovendor, Candler, NC (FHS), or from R&D Systems, Minneapolis, MN (NHS). Each study reported CV's $< 14\%$.

Statistical analysis

The associations between genotypes and fetuin-A level were analyzed within each cohort using linear regression in an additive model. All analyses were adjusted for age, sex, eigenvectors for population stratification, and if applicable, field center. Analyses were conducted separately for European and African Americans. See details for study-specific methods in Supplementary Material, Tables S1 and S2.

To combine results across cohorts, we performed an inverse variance–weighted meta-analysis using the software package METAL (38). Cohort-specific standard errors were adjusted using genomic control. In GWAS, we chose $P = 5 \times 10^{-8}$ as the threshold for significance (39). To investigate whether the multiple SNPs associated in the respective region were due to linkage disequilibrium (LD) with the top SNP or if multiple independent signals existed, we performed a meta-analysis based on models conditioned on the SNP with the smallest *P*-value.

To assess the variation in fetuin-A levels explained by our top SNPs, we compared the r^2 values from a model with just basic covariates versus a model that also included rs4917.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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