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ORIGINAL ARTICLE

Epidemiology/Genetics

Genetic dissection of serum vaspin highlights its causal role in lipid metabolism

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Abstract

Objective: Vaspin (visceral adipose tissue derived serine protease inhibitor, *SERPINA12*) is associated with obesity-related metabolic traits, but its causative role is still elusive. The role of genetics in serum vaspin variability to establish its causal relationship with metabolically relevant traits was investigated.

Methods: A meta-analysis of genome-wide association studies for serum vaspin from six independent cohorts (N = 7446) was conducted. Potential functional variants of *vaspin* were included in Mendelian randomization (MR) analyses to assess possible causal pathways between vaspin and homeostasis model assessment and lipid traits. To further validate the MR analyses, data from Genotype-Tissue Expression (GTEx) were analyzed, *db/db* mice were treated with vaspin, and serum lipids were measured.

Results: A total of 468 genetic variants represented by five independent variants (rs7141073, rs1956709, rs4905216, rs61978267, rs73338689) within the *vaspin* locus were associated with serum vaspin (all $p < 5\times10^{-8}$, explained variance 16.8%). MR analyses revealed causal relationships between serum vaspin and triglycerides, low-density lipoprotein, and total cholesterol. Gene expression correlation analyses suggested that genes, highly correlated with *vaspin* expression in adipose tissue, are enriched in lipid metabolic processes. Finally, *in vivo* vaspin treatment reduced serum triglycerides in obese *db/db* mice.

Conclusions: The data show that serum vaspin is strongly determined by genetic variants within *vaspin*, which further highlight vaspin's causal role in lipid metabolism.

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INTRODUCTION

The adipokine vaspin (visceral adipose tissue derived serine protease inhibitor, *SERPINA12*) was first discovered in white adipose tissue (AT) of the Otsuka Long-Evans Tokushima Fatty rat when obesity and insulin resistance (IR) reached their peak in this model of abdominal obesity and type 2 diabetes (T2D) [1]. Vaspin is predominantly expressed in skin, but also in pancreatic islets, gastric cells, and hypothalamus, implying a pleiotropic role [2]. Serum vaspin strongly correlates with body mass index (BMI) and IR [3] in humans and is elevated in the prediabetic stage, whereas it declines during diabetes progression and body weight loss [4]. Vaspin administration improves glucose tolerance and insulin sensitivity (IS) [1] and reduces food intake in obese mice [4]. Despite the strong evidence for vaspin's beneficial effects on glucose metabolism [5], the underlying molecular metabolic pathways are poorly understood.

Particularly important is the search for corresponding proteases inhibited by vaspin. One plausible target protease is kallikrein7 [6], which is able to cleave and degrade the A and B chains of insulin [6, 7]. By inhibition of kallikrein7 via the classical serpin mechanism, vaspin diminishes insulin degradation in blood [6]. Both proteins have also been found to be co-expressed in murine pancreatic β-cells [6]. It is conceivable that an improved glucose metabolism upon vaspin treatment in mice could be attributed to the increase of plasma insulin concentrations [6]. Another mode of action was described by Nakatsuka et al. [8] favoring the potential of vaspin to amend endoplasmic reticulum (ER) stress in obesity by building an anti-inflammatory cell surface receptor complex with GRP78/MTJ-1 in the liver [8]. Furthermore, vaspin serves as a ligand for a cell surface GRP78/voltage-dependent anion channel complex in endothelial cells that exerts anti-apoptotic, proliferative, and protective effects on vascular walls in diabetic rats [9]. Finally, vaspin is assumed to protect against vascular endothelial cell damage through mediation of the PI3K-Akt pathway, thus serving to ameliorate atherosclerosis [10, 11].

Given the physiological relevance of vaspin and its therapeutic potential in the treatment of IR/T2D in obesity, understanding the mechanisms underlying the variability of serum vaspin concentrations has proved to be highly relevant and in turn, has motivated several genetic studies. Although Kempf et al. presented the single-nucleotide polymorphism (SNP) rs2236242 in the vaspin gene with AA-genotype conveying an increased risk for T2D [12], a genome-wide association study (GWAS) on serum vaspin including 826 participants revealed several SNPs within or near the vaspin locus on chromosome (chr.) 14 [13]. Although the functional polymorphisms have not been identified in these studies, it has been shown by Teshigawara et al. that 7% of the Japanese population carrying the minor AA-genotype of the SNP rs77060950 (serum vaspin levels CC [0.6 ± 0.4 ng/mL], CA [18.4 ± 9.6 ng/mL], AA [30.5 ± 5.1 ng/mL]) present significantly elevated levels of serum vaspin [14]. In line with this, we have recently found rs76624128, which is in strong linkage disequilibrium (LD) with rs77060950, to be associated with up to five-fold increased higher serum vaspin concentrations in ~1% of the Sorbs, a Slavonic minority in Germany (data not shown).

Study Importance

What is already known?

- Vaspin displays various pleiotropic effects on obesity-related metabolic disorders, but causal relationships are poorly understood.
- Genetic variants in the vaspin locus associate with circulating vaspin concentrations.

What does this study add?

- Genetic mediation analyses suggest that vaspin affects lipid metabolism including triglycerides, low-density lipoprotein, and total cholesterol.
- Genes whose expression highly correlates with *vaspin* expression in adipose tissue are enriched in lipid metabolic processes.
- Vaspin treatment reduces serum triglycerides in obese *db/db* mice.

How might these results change the direction of research or the focus of clinical practice?

- Taking into account the therapeutic potential of vaspin in treatment of metabolic sequelae of obesity as has previously been suggested in animal models, the present data might be of eminent interest for researchers as well as clinicians in the field of metabolic diseases such as obesity.

The current knowledge on vaspin genetics is still inconclusive and warrants further analyses. Therefore, we conducted a genetic study for serum vaspin by integrating six GWAS from independent European studies (N = 7446) to identify genetic variants contributing to the variability of serum vaspin and to narrow down potential causal variants. Additionally, independent variants were included in Mendelian randomization (MR) analyses to assess possible causal chains between vaspin and metabolically relevant traits. Furthermore, *in vivo* studies in *db/db* mice were conducted to complement the GWAS findings.

METHODS

Participants

Sorbs cohort

Participants from the self-contained population of Sorbs in Germany were recruited and extensively phenotyped [15]. A sample set of 842 Sorbs with complete high-quality genotype, covariate, and phenotype information was included in this study.

KORA

The Cooperative Health Research in the Region of Augsburg (KORA) study is a series of independent population-based epidemiological surveys and follow-up studies of participants living in the region of Augsburg, Germany [16]. The present project was based on KORA follow-up study F4 (2006–2008). A total of 1426 nondiabetic participants [17] were included in the present study and genotyped for genetic analyses.

Prevalence, Prediction and Prevention of diabetes (PPP)-Botnia Study

The PPP-Botnia Study is a population-based study from the region of Botnia, Finland [18]. In the present study, 337 participants with normal glucose tolerance from PPP that also participated in the Diabetes Genetics Initiative (DGI) were included [19].

LIFE-Heart

LIFE-Heart is an observational study recruiting individuals with suspected coronary artery disease due to clinical symptoms (noninvasive testing), with confirmed stable coronary artery disease or myocardial infarction [20]. The present study included 941 individuals.

LIFE-Adult

LIFE-Adult is a population-based cohort consisting of 10,000 randomly selected but age- and sex-stratified inhabitants of the city of Leipzig, Germany [21]. Here, 3010 participants have been included.

Study of Health in Pomerania (SHIP-TREND)

A population-based, stratified (according to age, sex and city/country of residence) random sample of 8826 adults aged 20–79 years was drawn from population registries and facilitated by centralization of local population registries in the Federal State of Mecklenburg-West Pomerania, Germany. In the present study, 895 participants were included.

The analyses were restricted to participants with normal glucose tolerance (NGT), at least for those cohorts where impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) status was available (Sorbs, PPP-Botnia, KORA). Unfortunately, inclusion of participants with IGT or IFG cannot be ruled out in cohorts with self-reported diabetes status. Because of the missing IGT and IFG status in these participants, it was not possible to check whether restricting to NGT would have altered the results. For the LIFE cohorts, participants with T2D have been excluded (defined as HbA1c > 6.5%, medication with the antidiabetic drug ATC A10 or anamnestic information). All participants gave written informed consent before taking part. All studies meet the ethical standards of the Declaration of Helsinki and have been approved by their ethical committee (University of Leipzig Sorbs: Reg. No 088–2005; LIFE-Heart: Reg. No 276/05-ek and ClinicalTrials.gov No. NCT00497887; LIFE-Adult: Reg. No 263-2009-14122009). Detailed information for all cohorts is given in Table S1.

Measurement of serum vaspin

Briefly, to collect the blood samples, we used commercially available whole-blood EDTA tubes to suppress blood coagulation. In addition, we used a second blood tube for serum and took off the according fraction and stored it immediately at −80°C until serum vaspin measurement. In the Sorbs, PPP-Botnia, SHIP-TREND, and KORA cohorts, serum vaspin was measured using a commercially available enzyme-liked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (AdipoGen, Seoul, Korea). The sensitivity of the assay is 12 pg/mL, and the intra- and interassay coefficients of variance were 1.3%–3.8% and 3.3%–9.1%, respectively. Blood samples were taken in the morning after an overnight fast and were stored at −80°C until analyses.

In LIFE-Heart and LIFE-Adult, vaspin concentrations were determined using the commercially available ELISA kit (Mediagnost, Reutlingen, Germany) with the DSX automatic ELISA test system. The sensitivity of the assay is 0.004 ng/mL, and the intra- and interassay coefficients of variance were 1.4–3.8 and 4.5–8.6%. LIFE-Adult but not LIFE-Heart samples were taken after an overnight fast. Both study samples were stored at −80°C until analyses.

Measurements of insulin, glucose, and lipid parameters in the LIFE cohorts

The outcome variables included in the MR analyses have been determined in the LIFE-Adult and LIFE-Heart cohorts using the following methods: for homeostasis model assessment (HOMA) insulin was measured by the electrochemiluminescence immunoassay (ECLIA) and glucose by photometric assay, both on Roche COBAS 8000. Triglycerides (TG), as well as low-density lipoprotein cholesterol (LDL-chol), and total cholesterol were measured by photometric assay on Roche COBAS 8000, too. All measurements have been performed in the Institute of Laboratory Medicine using accredited methods.

Statistical methods

The information of conducted statistical methods concerning (1) GWAS, (2) file quality control (QC), (3) meta-analysis of GWAS; (4) secondary analyses, (5) heritability estimates and co-localizations of GWAS signals and tissue gene expression quantitative trait loci (eQTL); (6) colocalization analysis; (7) MR; (8) STROBE-MR checklist of recommended items to address in reports of MR studies; and

(9) gene expression correlation analyses (GECAs) are provided in the Supplemental Method S1.

Animal experiments

Because the initial publication describing vaspin for the first time suggested its beneficial effects on glucose metabolism and potentially insulin sensitivity only in the challenged obese and insulin resistant state, we used female leptin-deficient *db/db* mice as these develop significant IR (in addition to obesity) [22].

db/db mice (Taconic Bioscience, Denmark) were housed in pathogen-free facilities (3–5 mice per group and cage) at 23°C on a 12 h light/dark cycle. All mice were fed a standard chow diet (EV153, 3.3% from fat, Ssniff, Germany) and had ad libitum access to water and food. At 12 weeks of age, animals (N = 6/6) were treated intraperitoneally (i.p.) with phosphate-buffered saline (PBS) or vaspin (2 mg/kg, once daily at 6 am) for 4 weeks. The recombinant vaspin was expressed, purified, and tested for biological activity as previously described [23]. Briefly, after immobilized metal-affinity chromatography and size-exclusion chromatography, identity was verified by Western blot and mass spectrometry, and purity was assessed by HPLC (always >95%). Biological activity was confirmed for every lot by complex formation assay with target protease kallikrein7 [6].

At the age of 16 weeks, fasted mice were sacrificed 1 day following the final vaspin injection. Serum concentrations of free fatty acids (FFA), TG, total as well as LDL and high-density lipoprotein cholesterol (HDL)-chol were measured as described before [24]. Statistical significance was evaluated by multiple unpaired *t*-tests corrected for multiple comparisons using the Holm-Šídák method. All animal experiments were approved by the local authorities of the State of Saxony, Germany (Landesdirektion Leipzig, TVV29-08).

RESULTS

Meta-analysis of GWAS for serum vaspin

The estimated heritability of serum vaspin was 59.3% (95% CI: [36.0%; 82.6%], $p = 6.14 \times 10^{-7}$ adjusted for age, sex, and BMI) in the LIFE-Adult and LIFE-Heart cohorts. The heritability in 1965 female participants reached 69.3% (95% CI: [24.4%; 100%], $p = 0.0025$) and in 1998 male participants, 80.2% (95% CI: [32.4%; 100%], $p = 9.96 \times 10^{-4}$) (both adjusted for age and BMI).

No general inflation of meta-analysis statistics in the meta-GWAS of six studies (7446 participants in total) was observed ($\lambda = 1$ for fixed effect model of all, males and females). A total of 468 SNPs, all within

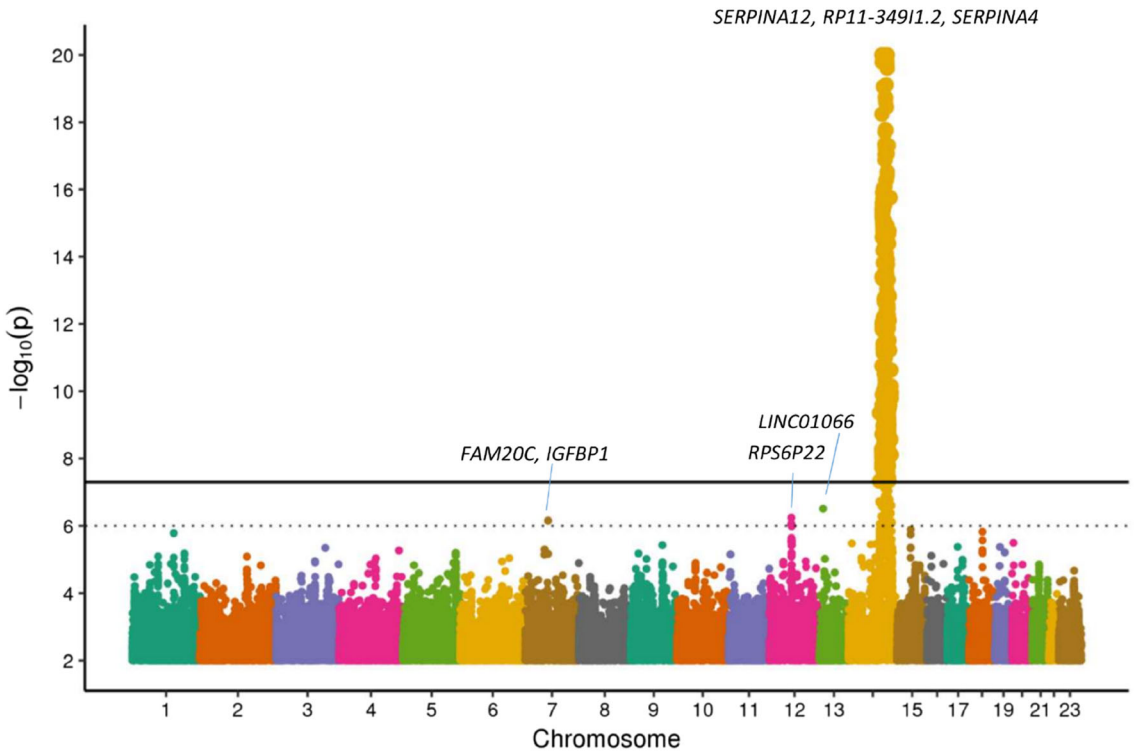

FIGURE 1 Manhattan plot of serum vaspin meta-genome-wide association study of six cohorts (N = 7446). Lines correspond to genome-wide ($p < 5 \times 10^{-8}$, solid line) and suggestive ($p < 1 \times 10^{-6}$, dotted line) statistical significance. Nearest genes are provided per locus. The *y*-axis is truncated at a value of 20. The lowest *p*-value of 7.36×10^{-298} was detected for rs61978271 at chromosome 14.

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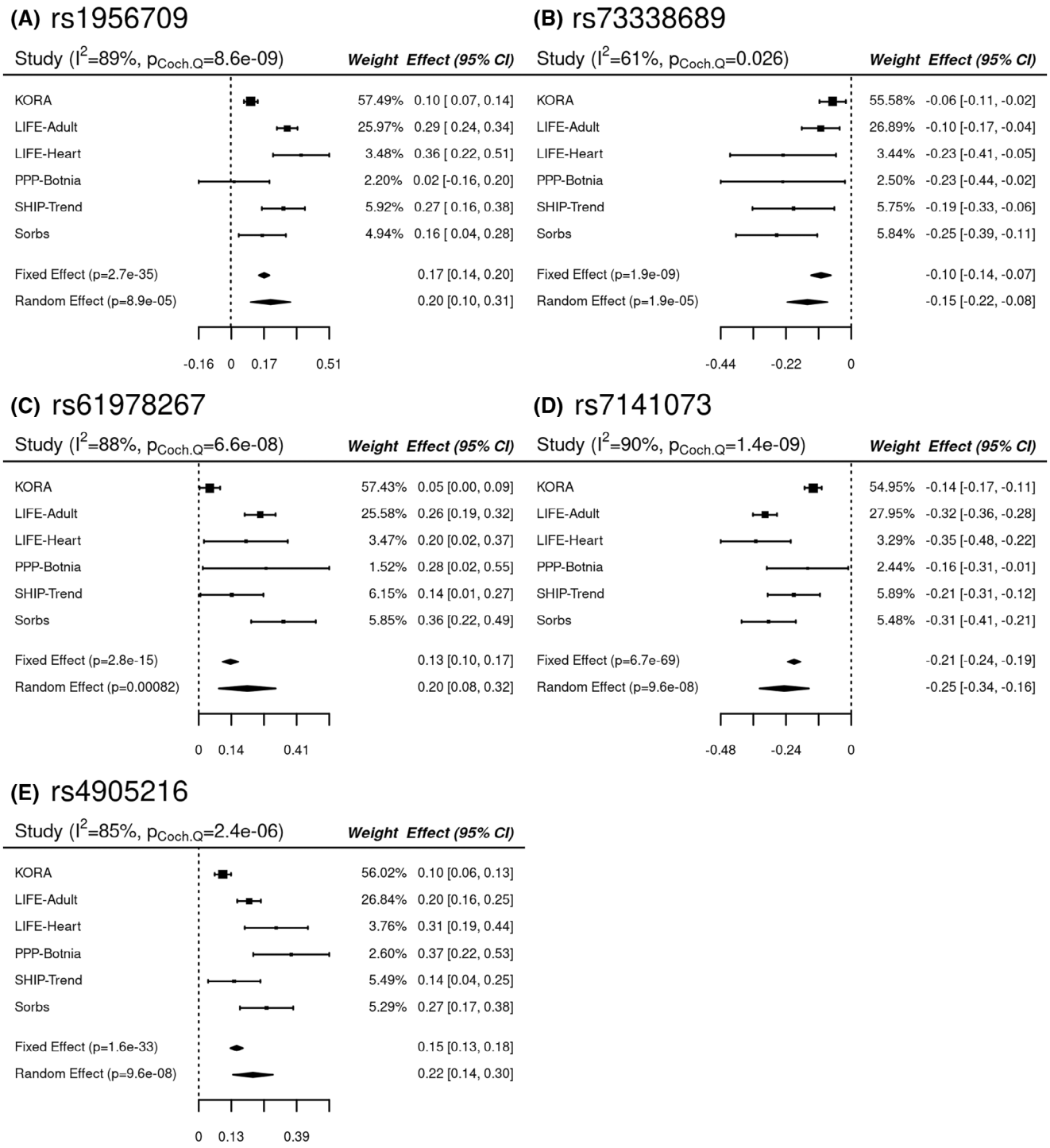

FIGURE 2 Forest plots of independently associated signals. We present unconditional statistics.

one locus on chr.14 (14q32.12, 14q32.13) reached genome-wide significance ($p < 5 \times 10^{-8}$), (Figure 1–3; Table S2). The $p = 7.36 \times 10^{-298}$ was detected for rs61978271 (Figure 1, Table S2). This SNP was imputed. The strongest genotyped variant in LIFE-Adult and LIFE-Heart was rs17091005 (LIFE-Adult: $p = 5.6 \times 10^{-156}$, LIFE-Heart: $p = 4.7 \times 10^{-33}$). Conditional and joint (COJO) analysis revealed five independent variants (rs7141073, rs1956709, rs4905216, rs61978267, rs73338689) (Table 1). All of them showed consistent effect directions across studies (Figure 2). Credible sets represented by these variants comprised 20 polymorphisms in total (Table 2). Only one of these variants (rs17094914) showed a notable deleteriousness (CADD = 10; Method S2). This SNP was extracted from the credible set of rs73338689 and is located within the intron of *SERPINA12*. According to the HaploReg database, this variant

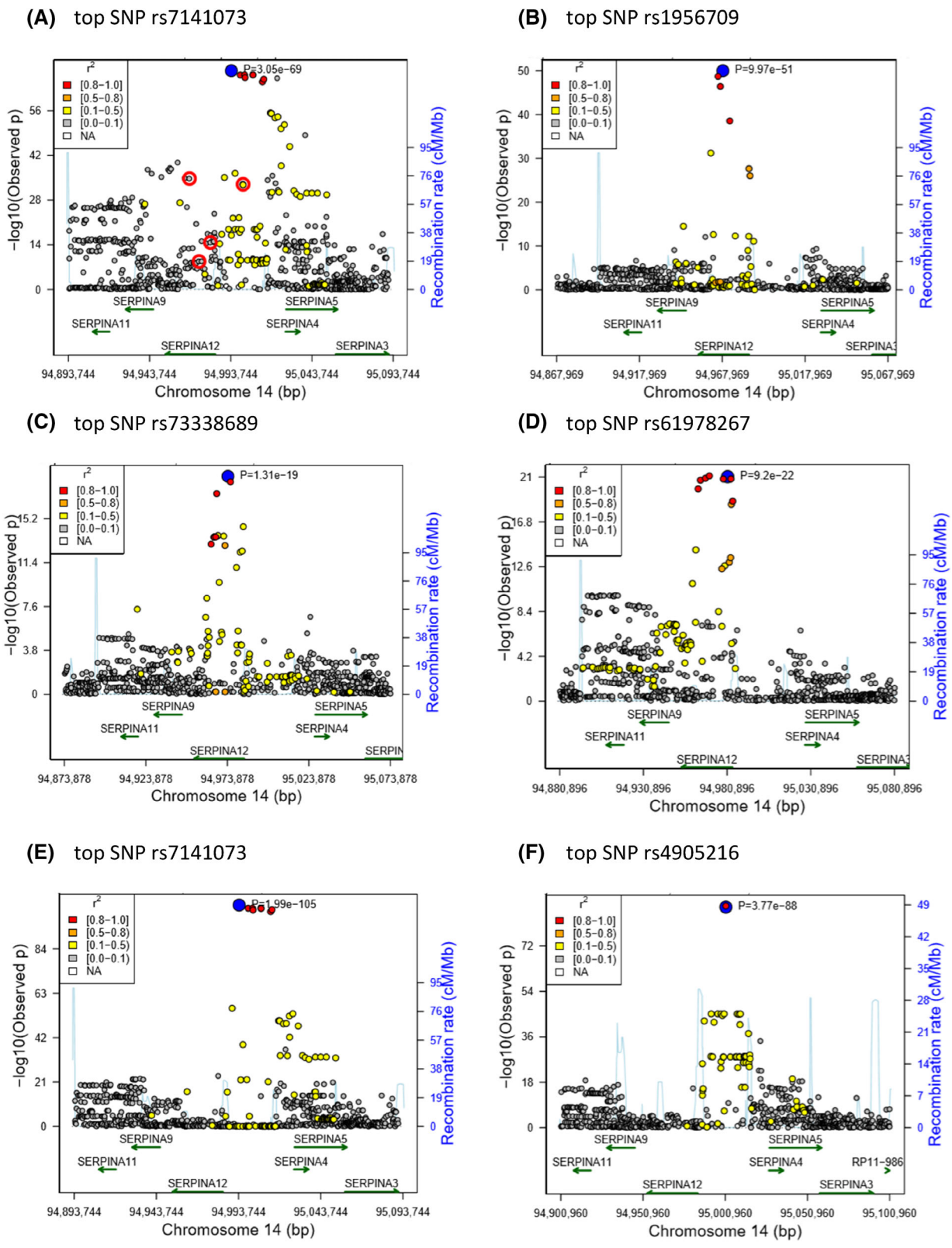

FIGURE 3 Regional association (RA) plots of the 14q32.13 (*SERPINA12*) locus. (A) RA plot of the unconditional statistics. The blue circle corresponds to the top associated variant, and the red circles represent additional independent variants. (B–F) RA plots of conditional statistics of each of the five independent variants. Effects are adjusted for the respective other four variants using conditional and joint (COJO) analyses. SNP, single-nucleotide polymorphism.

TABLE 1 Independently associated genetic variants at *SERPINA12* locus according to COJO analysis

SNP	Pos.	Effect allele/ other allele	Nearest genes of functional relevance (distance to SNP)	Explained variance SNP	Beta fixed (*p*-value)	I^2 (%)	Beta random (*p*-value)	Conditional beta (*p*-value)	*p*-value (sex difference)	Correlating GWAS (r^2 + trait)
rs7141073	94993744	T/C	SERPINA12 (9.6 kb); SERPINA4 (34 kb); SERPINA5 (34 kb); SERPINA9 (48 kb)	0.0635	−0.21 (6.7×10^{-69})	90	−0.25 (9.6×10^{-8})	**−0.27 (2.0×10^{-105})**	0.672	0.56 vaspin levels; 0.526 blood protein levels
rs1956709	94967969	G/A	SERPINA12 (0 kb); SERPINA9 (22 kb); SERPINA11 (49 kb); SERPINA4 (59 kb)	0.0292	0.17 (2.7×10^{-35})	89	0.20 (8.9×10^{-5})	**0.21 (10.0×10^{-51})**	0.493	NA
rs4905216	95000960	C/G	SERPINA12 (17 kb); SERPINA4 (26 kb); SERPINA5 (27 kb); SERPINA9 (55 kb)	0.0524	0.15 (1.65×10^{-33})	85	0.22 (9.6×10^{-8})	**0.25 (3.8×10^{-88})**	0.651	NA
rs61978267	94980896	T/C	SERPINA12 (0 kb); SERPINA9 (35 kb); SERPINA4 (47 kb); SERPINA5 (47 kb)	0.0120	0.13 (2.8×10^{-15})	88	0.20 (8.2×10^{-4})	**0.16 (9.2×10^{-22})**	0.348	NA
rs73338689	94973878	C/G	SERPINA12 (0 kb); SERPINA9 (28 kb); SERPINA4 (54 kb); SERPINA5 (54 kb)	0.0111	−0.10 (1.9×10^{-9})	61	−0.15 (1.9×10^{-5})	**−0.15 (1.3×10^{-19})**	0.176	0.55 obesity-related traits

Note: Conditional statistics are shown in bold. Beta estimates correspond to log-changes of vaspin levels. The last column reports linkage disequilibrium (LD) with other GWAS traits as reported in the GWAS catalog.
Abbreviations: COJO, conditional and joint; GWAS, genome-wide association study; I^2, imputation quality of imputed SNPs according to IMPUTE2; NA, not available; Pos., genomic position; SNP, single-nucleotide polymorphism.

represents four eQTL hits and four altered regulatory motifs (Myb_1, Myb_3, RXR::LXR, RXRA_known6). The SNP conveys an eQTL for *SERPINA9* and *SERPINA12* in skin according to Genotype-Tissue Expression (GTEx) and for *SERPINA1* in blood [25].

Furthermore, three loci on chr.7 near *FAM20C* and *IGFBP1*, on chr.12 (*RPS6P22*), and on chr.13 (*LINC01066*) reached suggestive evidence for association with $p < 1 \times 10^{-6}$ (Figure 1, Table S2).

Sex-stratified analyses confirmed the major locus on chr.14 but did not reveal additional genome-wide significant loci (Figure 4). None of the independent variants showed significant differences in effect estimates between sexes.

In females (N = 3732), we detected three suggestive associations with SNPs on chr.9 (*TXNDC8*), chr.15 (*RORA*) and chr.2 (*RP11-444A22.1*) (Figure 4A), whereas in males (N = 3561), five suggestive loci were detected (Figure 4B). Seventeen SNPs were located on chr.9 (one LD-cluster, nearest gene: *TRPM3*), three on chr.19 (one LD-cluster, nearest gene: *SCGB2B2*), two on chr.7 (two LD-cluster, nearest genes: *FAM20C* and *IGFBP1*), two on chr.3 (one LD-cluster, *KCNMB2*), and one on chr.4 with the nearest gene *FAM13A* (Figure 4B, Table S2).

Comparison with GWAS catalog and eQTL colocalization analysis

The GWAS catalog (https://www.ebi.ac.uk/gwas/home; downloaded at 2022/10/05) reports only one study when searching "serum vaspin" [13] and two variants by using "vaspin measurement" [26], namely, rs11160190 mapping between *SERPINA4* and vaspin [13] and rs8006968 mapping to the *SERPINA12*/vaspin gene. Furthermore, by searching "*SERPINA12*," 20 listed SNPs published in 13 studies were found. Out of these, three published analyses presented serum vaspin levels as a related trait [13, 26, 27]. Fifteen out of the 20 listed SNPs appear among the associated variants in our meta-analysis (Table S2). All results can be found in Table S3.

Using conditional statistics, the independent hits were subjected to colocalization analyses with eQTLs of *SERPINA12*. A colocalization was observed for rs1956709 (Posterior Probability for hypothesis H_4 [PP4] = 94%) and with rs61978267 (PP4 = 63%) in "skin not sun exposed suprapubic." It should be noted that in GTEx, gene expression of *SERPINA12* was only reported for skin tissue.

MR reveals a causal link between vaspin and lipid traits

We applied MR analyses to clarify the causal relationships of vaspin with metabolic traits. Based on previously reported correlations with vaspin [28], we focused our analyses on HOMA, TG, LDL-chol and total cholesterol (see respective correlations in LIFE-Adult

TABLE 2 99% credible sets of independently associated variants

SNP	CADD	DANN	Eigen	EigenPC	GWAVA Region	GWAVA TSS	GWAVA Unmatched	Regulome score	PostProb	SumProb
rs1956709	**3.537**	**0.749**	**0.077**	**−0.165**	**0.19**	**0.20**	**0.28**	**5**	**0.938**	**0.938**
rs7161421	2.893	0.172	−0.115	−0.190	0.19	0.10	0.18	5	0.062	1.0
rs73338689	**0.248**	**0.659**	**−0.195**	**−0.192**	**0.30**	**0.25**	**0.18**	**6**	**0.700**	**0.700**
rs17094914	10.120	0.751	0.488	−0.088	0.41	0.48	0.11	5	0.266	0.966
rs8015166	2.623	0.654	0.018	−0.152	0.25	0.17	0.24	6	0.032	0.998
rs61978264	5.508	0.698	0.136	−0.150	0.19	0.23	0.43	5	0.250	0.250
rs61978267	**3.076**	**0.794**	**−0.133**	**−0.196**	**0.24**	**0.23**	**0.15**	**7**	**0.203**	**0.454**
rs12436152	3.619	0.804	−0.158	−0.202	0.26	0.32	0.25	5	0.152	0.606
rs77515403	6.363	NA	NA	NA	NA	NA	NA	NA	0.139	0.744
rs61978269	0.969	0.516	−0.356	−0.210	0.11	0.25	0.43	5	0.128	0.872
rs12433651	6.775	0.875	0.230	0.048	0.25	0.15	0.17	3a	0.106	0.978
rs8012043	1.159	0.429	−0.065	−0.183	0.20	0.26	0.15	5	0.020	0.997
rs7141073	**4.159**	**0.653**	**−0.486**	**−0.231**	**0.20**	**0.11**	**0.02**	**7**	**0.854**	**0.854**
rs11354340	0.920	NA	NA	NA	NA	NA	NA	NA	0.039	0.893
rs1951206	2.802	0.637	−0.249	−0.137	0.29	0.25	0.09	4	0.036	0.929
rs2402476	0.016	0.591	−0.982	−0.297	0.33	0.16	0.04	7	0.032	0.961
rs8013001	1.201	0.552	−0.676	−0.238	0.27	0.39	0.18	5	0.026	0.986
rs11160191	0.370	0.456	−0.560	−0.245	0.19	0.17	0.03	6	0.008	0.994
rs11306270	2.382	NA	NA	NA	NA	NA	NA	NA	0.737	0.737
rs4905216	**0.902**	**0.712**	**−0.321**	**−0.215**	**0.26**	**0.27**	**0.06**	**7**	**0.262**	**1.0**

Note: Single-nucleotide polymorphisms (SNPs) highlighted in bold represent the independent variants identified by conditional and joint (COJO) analysis. We present the respective 99% credible sets (separated by double rules) including their Posterior Probability (PostProb) and estimated deleteriousness (SumProb). Score information is provided in the Supplementary file Method S2.
Abbreviations: CADD, Combined Annotation Dependent Depletion; DANN, deleterious annotation of genetic variants using neural networks; EigenPC, Eigen score representing the first principal component of the covariance matrix; GWAVA, genome-wide annotation of variants by classifier (region score, transcription start site [TSS] score and an Unmatched score); NA, not available.

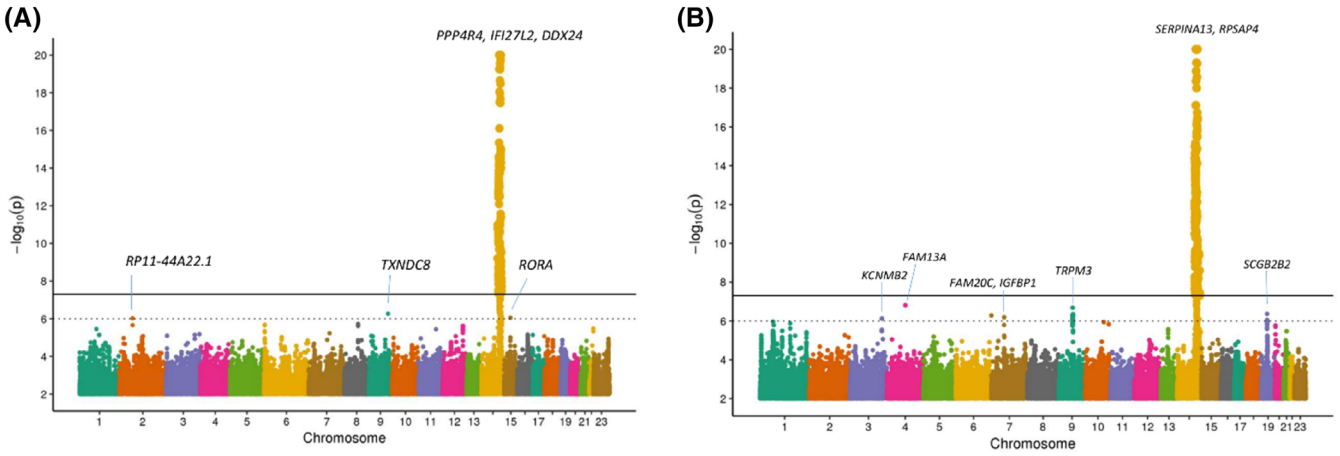

FIGURE 4 Manhattan plots of serum vaspin meta-genome-wide association study of six cohorts separated by sex: (A) females, N = 3732 and (B) males, N = 3561. Lines correspond to genome-wide ($p < 5 \times 10^{-8}$) and suggestive ($p < 1 \times 10^{-6}$) statistical significance. Nearest genes are provided per locus. The *y*-axis is truncated at a value of 20.

Figure 1 in Methods S1). MR analysis was performed using the five independent SNPs from the COJO analysis as instruments of serum vaspin. Single SNP results are shown in Table S4. Using the inverse-variance method, we detected a causal effect of vaspin on TGs ($p = 0.031$), LDL-chol ($p = 0.015$), and total cholesterol ($p = 0.009$) (Table 3).

No evidence for a causal relationship with HOMA was detected ($p = 0.56$). Sensitivity analysis neglecting two LD partners

TABLE 3 MR analyses

Outcome	Beta	SE	95% confidence interval of beta	*p*-value
HOMA	−0.0049	0.0085	[−0.022, 0.012]	0.56
TG	−0.0062	0.0029	[−0.012, −0.00058]	**0.031**
Chol	−0.0074	0.0028	[−0.013, −0.0019]	**0.0088**
LDL-chol	−0.0081	0.0033	[−0.015, −0.0016]	**0.015**

Note: We analyzed the causal relationship of vaspin on homeostasis model assessment (HOMA), triglycerides (TG), total cholesterol (Chol), and low-density lipoprotein cholesterol (LDL-chol) by MR. Evidence of the five vaspin variants rs7141073, rs1956709, rs4905216, rs61978267, and rs73338689 were summarized with the IVW method correcting for residual linkage disequilibrium (LD) between variants. *p*-values < 0.05 were highlighted in bold.
Abbreviations: Beta, MR effect representing changes of outcome variable per log-change of vaspin; MR, Mendelian randomization; SE, standard error of MR effect.

(LD $r^2 = 0.3$) confirmed the findings. Detailed results are shown in Table S5. Results of alternative MR methods are presented as Figure S1 and confirmed the findings.

GECA suggests involvement in lipid metabolic processes

GECA using the GTEx database revealed 492 genes whose expression correlated with vaspin expression in both ATs and skin ($p < 0.001$ from 1000 random samplings considering 20,000 expressed genes; Table S6). A gene ontology enrichment analysis of these genes revealed gene ontology (GO) categories related to skin development, as well as lipid metabolic processes (Figure S2).

Treatment of obese *db/db* mice with recombinant vaspin improves serum TG levels

Because the aforementioned genetic analyses clearly suggest the role of vaspin in lipid metabolism, we validated these effects *in vivo* by treating *db/db* mice with recombinant human vaspin. Although treatment had no significant effect on body weight and blood glucose levels (data not shown), serum TGs were significantly reduced (Figure 5). In addition, there was a trend toward lower FFAs ($p = 0.1$), whereas total cholesterol, as well as HDL- and LDL-chol, remained unchanged (Figure 5).

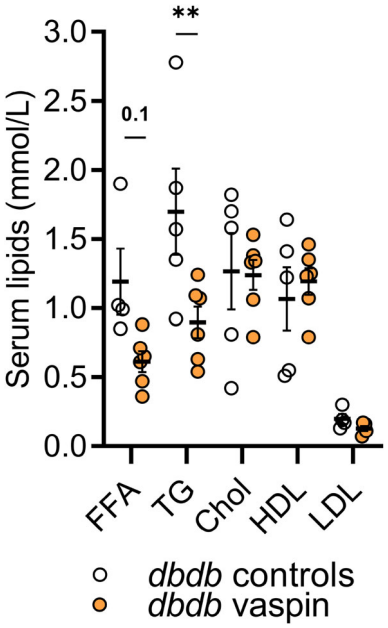

FIGURE 5 Vaspin reduced serum triglycerides (TG) in genetically obese *db/db* mice after 4 weeks of treatment with recombinant vaspin (2 mg/kg, i.p., daily; controls received PBS). Serum lipid profile after treatment revealed significantly reduced serum TG levels, and a trend for lower free fatty acids (FFA). Cholesterol (Chol) and high- and low-density lipoprotein cholesterol (HDL and LDL, respectively) were unchanged. Statistical significance was evaluated by multiple unpaired *t*-tests corrected for multiple comparisons using the Holm-Šídák method. ** indicated $p < 0.01$.

DISCUSSION

We employed genetic analyses to investigate the role of genetic variation in circulating vaspin and to examine potential causal relationships between vaspin and metabolic traits related to obesity and T2D. We conducted a meta-GWAS on serum vaspin in 7446 participants from six independent cohorts of European ancestry. The estimated heritability of vaspin with 59.3% indicated a strong genetic background, which is in close agreement to that of Tarabeih et al. [29]. However, this estimate is the so-called narrow sense heritability, which is captured by the analyzed SNP panel, and so the total heritability is likely to be higher. In total, 468 SNPs within the vaspin locus on chr.14 were significantly associated with circulating vaspin. Five independent SNPs (rs7141073, rs1956709, rs4905216, rs61978267, rs73338689) indicated a considerable locus heterogeneity. Moreover, MR analysis including the five representative SNPs revealed causal relationships between serum vaspin and TGs as well as LDL-chol and total cholesterol. GECAs suggested that genes, highly correlating with vaspin expression in AT, are enriched in lipid metabolic processes. The genetic analyses pointed to the causal role of vaspin in lipid metabolism, which was finally validated *in vivo* by showing that chronic vaspin treatment reduced serum TGs in genetically obese *db/db* mice.

Consistent with the previously reported small single-study vaspin GWAS [13], we confirmed the *SERPINA12-RP11-349I1.2-SERPINA4* genetic locus conveying the strongest association with circulating vaspin. The findings highlight the vaspin locus on chr.14 as the major genetic driver controlling vaspin concentrations [13]. The SNP showing the strongest association is located 3.4 kb upstream of the vaspin and does not exhibit any sex dimorphism, although higher vaspin concentrations in women [3] compared with men have previously been reported [30]. Based on previous as well as our present data, this

locus likely harbors a functional variant in or near the vaspin gene. Indeed, the previously acknowledged functional variant rs76624128 was ranked as number 16 of the top associated SNPs reaching a *p*-value of 1.41×10^{-108}. The SNP is nearly in complete LD with rs77060950, whose minor A-allele was suggested to be responsible for dramatically increased serum vaspin in 7% of the Japanese population [14]. Rs76624128 is present in ~1% of the European population, in which the minor G-allele carriers have a significantly higher mean serum vaspin than wild-type A-allele carriers (25.9 ± 11.3 ng/mL vs. 1.1 ± 3.4 ng/mL) [14]. However, because five independent variants were detected in the present study, other causal variants most likely exerting a regulatory role in transcription cannot be ruled out. Indeed, for one of the loci with rs1956709 as the index variant, we detected evidence of colocalization with an eQTL of *SERPINA12* in skin tissue suggesting that this variant regulates the expression of this gene. It needs to be pointed out that the expression of *SERPINA12* is low in tissues other than skin limiting the power for colocalization analyses. However, cis-eQTLs are usually overlapping between tissues (e.g., the top-eQTL in skin is also significant in liver) so that a genetic regulation of *SERPINA12* could be of relevance in other tissues as well.

By using these five independent variants as instruments in MR analysis, we found causal relationships between vaspin and several lipid traits, which further underline the supposed protective action of vaspin in metabolic diseases including the metabolic syndrome [28, 31] and particularly IR in the state of obesity [2, 28, 32]. This does not preclude the existence of other causal mediators in the causal path between vaspin and the analyzed lipid traits. Although the results for LDL-chol and total cholesterol were robust against multiple testing correction, the significance for the associations with TG would not withstand multiple testing corrections. However, the negative correlation between circulating vaspin and TG is also in line with previously reported findings in experimental models shown by Nakatsuka et al. [8]. Vaspin-transgenic mice were protected against diet-induced obesity, IR, and fatty-liver, whereas vaspin-deficient mice developed glucose intolerance due to the up-regulation of ER stress markers like ATF6, peIF2α, and pIRE1α in the liver [8]. In addition, the authors of this paper reported a significantly decreased hepatic TG content in high-fat-high-sugar–fed transgenic mice (vaspin$^{+/+}$ vs. vaspin$^{+/-}$ and vaspin$^{-/-}$), suggesting that the liver may be one of the major targets [8]. In a previous study, we observed positive correlation between serum vaspin and TGs, which was further supported by MR analyses suggesting a causal impact on LDL-chol [28]. In support of the metabolically beneficial action of vaspin in obesity [1, 2, 10, 28], the GECA and gene ontology enrichment analyses point out genes involved in lipid metabolic processes (e.g., low-density lipoprotein receptor). Ultimately, our *in vivo* studies validated the involvement of vaspin in lipid metabolism by presenting reduced TG and lower FFA levels in vaspin-treated mice. It is of note that we did only see strong effects of a chronic, yet still temporary, treatment with recombinant vaspin, on circulating TG levels, whereas there was no effect on cholesterol. Because of the high interanimal variability in the measurements of especially TG and cholesterol, the effect on cholesterol may be lost due to the small number of animals used in this experiment resulting in too little power. Along these lines, we have recently published the beneficial effects of transgenic overexpression of human vaspin in a mouse model on the frequently used C57BL6/N background [33]. These mice exhibit high levels of circulating human vaspin and in support of our findings here, we observed a significant reduction in total cholesterol levels after feeding an HFD, whereas in this model, circulating TG were not different. Yet there also was a trend for lower TG under chow conditions in the vaspin-transgenic mice. Finally, no causal relationship between vaspin and HOMA was detected in mediation analyses. Because sample sizes of genome-wide association meta-analyses for HOMA are still much smaller compared with the considered lipid traits, we cannot exclude that this negative finding is due to lack of power.

Along with the locus on chr.14, we further detected suggestive associations within the chr.7 (*FAM20C-IGFBP1* locus), on chr.12 (*RPS6P22*), and on chr.13 (*LINC01066*) proposing potentially novel genetic regulators of serum vaspin but requiring validation in further studies. Interestingly, the *FAM20C* is supposed to play a role in biomineralization and lipid homeostasis [34], which is in line with findings in vaspin overexpressing transgenic mice suggesting vaspin's involvement in lipid metabolism [8]. Further support comes from correlations of vaspin with lipid traits in humans [28] and from the causal link between vaspin and lipid traits in MR analysis presented here. Further studies addressing vaspin functionality are warranted to clarify whether *FAM20C* might act as a regulator of vaspin, which in turn would mediate the described role of *FAM20C* in lipid metabolism. The second gene mapping to chr.7, the *IGFBP1* (insulin-like growth factor binding protein 1; 9-kb distance) encodes a circulating protein mainly expressed in the liver, and binding both insulin-like growth factors (IGFs) I and II. Thereby it prolongs their half-lives and alters the interaction with cell surface receptors. The IGFBP1-protein is involved in cell migration and metabolism, showing associations with IGT, vascular disease, and hypertension in humans [35], which underline vaspin's predicted protective role in inflammatory and insulin signaling pathways [1, 2].

There are evident sex-specific differences in vaspin concentrations with increased levels observed in females, which has to be considered in vaspin-related studies [3, 30]. Accordingly, we calculated the heritability of serum vaspin in both sexes and found lower heritability estimates in female than in male participants (69.3% vs. 80.2%). Although the sex-stratified GWAS did not detect sex-specific or sex-differential association signals reaching the thresholds for genome-wide significance, several sex-specific loci with suggestive associations reaching $p < 10^{-6}$ were found. Accordingly, in our meta-GWAS, we did not find any evidence of sex-differential effects of the independent variants of the chr.14. Large-scale GWAS providing higher statistical power than the present study will be required to robustly identify sex-dimorphic loci. This seems to be a very likely scenario as large genome-wide proteogenomic mapping with thousands of proteins/biomarkers in tens of thousands of individuals have recently emerged [36] and are paving new research avenues in genetic dissection of complex diseases. Nevertheless, studies like ours, which combine sophisticated bioinformatics pipelines in genetic analyses of complex traits with *in vivo* experiments and which allow to

validate connections between putative disease-associated genetic variants and medical conditions, will be essential to better understand molecular mechanisms underlying the observed genotype–phenotype associations. As exemplarily demonstrated by the present study, the suggested role of vaspin in lipid metabolism based on our genetic and bioinformatic analyses was successfully validated *in vivo* in vaspin-treated diabetic mice.

We are aware of limitations of the present study. In particular, there was a relatively small number of samples analyzed, which resulted in a limited statistical power to detect statistically significant associations. Also, in our meta-GWAS, we only considered European individuals. Thus, studies in other ethnicities are required to assess potential ethnic heterogeneity of our findings. Nevertheless, the strength of our study is the finding of a causal relationship of serum vaspin and parameters of the lipid metabolism underlined by animal experiments and epidemiology.

CONCLUSION

Our data suggest the vaspin locus as the major and most plausible genomic area harboring functional variants, although further in-depth functional studies are required to clearly identify the molecular mechanisms behind the observed associations. More importantly, by combining genetic analyses with *in vivo* experimental approaches, our study supports a causal relationship between vaspin and lipid traits, thus supporting its previously postulated protective role in lipid metabolism. O

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

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SUPPORTING INFORMATION

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