



Proteomic analysis of diabetes genetic risk scores identifies complement C2 and neuropilin-2 as predictors of type 2 diabetes: the Atherosclerosis Risk in Communities (ARIC) Study

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Abstract

Aims/hypothesis Genetic predisposition to type 2 diabetes is well-established, and genetic risk scores (GRS) have been developed that capture heritable liabilities for type 2 diabetes phenotypes. However, the proteins through which these genetic variants influence risk have not been thoroughly investigated. This study aimed to identify proteins and pathways through which type 2 diabetes risk variants may influence pathophysiology.

Methods Using a proteomics data-driven approach in a discovery sample of 7241 White participants in the Atherosclerosis Risk in Communities Study (ARIC) cohort and a replication sample of 1674 Black ARIC participants, we interrogated plasma levels of 4870 proteins and four GRS of specific type 2 diabetes phenotypes related to beta cell function, insulin resistance, lipodystrophy, BMI/blood lipid abnormalities and a composite score of all variants combined.

Results Twenty-two plasma proteins were identified in White participants after Bonferroni correction. Of the 22 protein–GRS associations that were statistically significant, 10 were replicated in Black participants and all but one were directionally consistent. In a secondary analysis, 18 of the 22 proteins were found to be associated with prevalent type 2 diabetes and ten proteins were associated with incident type 2 diabetes. Two-sample Mendelian randomisation indicated that complement C2 may be causally related to greater type 2 diabetes risk (inverse variance weighted estimate: OR 1.65 per SD; $p=7.0 \times 10^{-3}$), while neuropilin-2 was inversely associated (OR 0.44 per SD; $p=8.0 \times 10^{-3}$).

Conclusions/interpretation Identified proteins may represent viable intervention or pharmacological targets to prevent, reverse or slow type 2 diabetes progression, and further research is needed to pursue these targets.

Keywords Beta cell · Diabetes · Genetic risk score · Insulin resistance · Mendelian · Proteomics

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Research in context

What is already known about this subject?

- Genetic liability to type 2 diabetes is well-established
- Genetic risk scores have been developed to capture heritable liabilities for type 2 diabetes phenotypes, including insulin resistance, beta cell dysfunction, BMI and lipodystrophy

What is the key question?

- Using a large-scale agnostic proteomic approach, is it possible to identify proteins and pathways through which type 2 diabetes phenotype risk scores influence type 2 diabetes pathophysiology?

What are the new findings?

- We identified 22 novel proteins associated with genetic risk scores for type 2 diabetes, and most of these were found to be associated with prevalent or incident type 2 diabetes after adjustment for potential confounders
- Two-sample Mendelian randomisation analysis provided evidence that low levels of complement C2 and neuropilin-2 may be causally related to greater type 2 diabetes risk

How might this impact on clinical practice in the foreseeable future?

- Proteins identified through this genetic/proteomic approach may be viable targets for pharmacological or lifestyle interventions

Abbreviations

| | |
|------|-------------------------------------|
| ARIC | Atherosclerosis Risk in Communities |
| GRS | Genetic risk score |
| GWAS | Genome-wide association studies |
| IVW | Inverse variance weighted |
| MR | Mendelian randomisation |
| pQTL | Protein quantitative trait locus |

Introduction

Type 2 diabetes is characterised by complex disruptions in cell signalling and metabolic homeostasis across multiple organ systems. Among these, peripheral and hepatic insulin resistance, pancreatic beta cell dysfunction and adipose tissue accumulation are typical, and individuals with type 2 diabetes often present with various combinations of these phenotypes [1–3]. While lifestyle risk factors such as physical inactivity and hyper-caloric diets are well-established in disease development [4, 5], genetic liabilities also influence risk [6–9] and have been shown to contribute to the phenotypic heterogeneity of type 2 diabetes [9, 10].

Genome-wide association studies (GWAS) have identified over 450 gene variants related to type 2 diabetes [7–12]—most showing modest magnitudes of association with disease risk, typically <10% greater risk per allele [12–14]. Numerous additive genetic risk scores (GRS) for type 2 diabetes have been generated to integrate risk information across identified gene variants [6, 9]. Refining this approach to address the heterogeneity of

type 2 diabetes, Goodarzi et al [10] developed four type 2 diabetes GRS for the distinct phenotypes of beta cell function, insulin resistance, lipodystrophy and BMI/aberrant blood lipids, which were comprised of independent clusters of gene variants. These type 2 diabetes risk variants may have *cis* or *trans* effects on gene expression, and the proteins and networks through which they affect metabolic dysfunction are unknown.

The present study aimed to identify proteins and pathways through which type 2 diabetes risk variants may collectively influence pathophysiology. To strengthen causal inference, a stepwise approach was applied in which: (1) plasma proteins associated with the type 2 diabetes phenotype GRSs were identified from a platform comprised of 4870 protein measures; (2) cross-sectional and prospective associations with type 2 diabetes were examined for identified proteins; and (3) a two-sample Mendelian randomisation (MR) analysis was conducted to investigate potential causal relationships between these proteins and type 2 diabetes. Collectively, these findings aim to provide greater understanding of genetic liability to type 2 diabetes.

Methods

Study population The Atherosclerosis Risk in Communities Study (ARIC) of 15,792 individuals was originally designed to identify risk factors for cardiovascular disease and atherosclerosis and has been described in detail previously [15]. Male and female participants aged 45–64 years were recruited from four communities in the USA (Washington County,

MD; the northwest suburbs of Minneapolis, MN; Jackson, MS; Forsyth County, NC). Information about risk factors was obtained at baseline (visit 1; 1987–1989) and in follow-up examinations: visit 2 (1990–1992), visit 3 (1993–1995), visit 4 (1996–1998), visit 5 (2011–2013), visit 6 (2016–2017) and visit 7 (2018–2019). Visit 3 served as the baseline for this analysis. Information on risk factors and use of medications was also obtained through follow-up phone calls to participants (annually before 2012; twice-yearly thereafter). Institutional review boards at each site approved the study protocol, and written informed consent was obtained from all participants.

Genetic risk scores for type 2 diabetes GRS for type 2 diabetes were constructed from SNP dosages and clustered into four mutually exclusive phenotypes of beta cell function (50 SNPs), insulin resistance (29 SNPs), lipodystrophy (12 SNPs) and BMI/lipid abnormalities (12 SNPs), using SNPs and weights provided by Goodarzi et al [10]. Two additional GRS were generated from SNPs that remained uncategorised by Goodarzi et al in terms of diabetes phenotype (339 SNPs) and all SNPs combined (442 SNPs) [10]. For each participant, a weighted GRS was generated by multiplying the number of risk alleles at each variant by its corresponding diabetes-related risk value (logOR) and then summing the products. For variants unavailable in the ARIC GWAS data, a proxy variant in tight linkage disequilibrium ($r^2 \geq 0.80$) was substituted. In Black participants, no suitable proxy variants were available for rs145904381 (insulin resistance GRS) or rs1005752 (beta cell function GRS).

Genotyping Genotyping, imputation and quality control procedures have been described previously [16]. Genome-Wide Human SNP array 6.0 (Affymetrix, USA) was used to genotype genomic DNA extracted from whole blood, and race-specific imputation of variant dosages to the TOPMed reference panel (freeze 5b) was performed to increase the number of genetic markers beyond this array [17]. Individuals who were first-degree relatives of each other, genetic outliers and those whose genotypes did not match genotype data from other platforms were removed prior to imputation. Based on the GWAS data, principal components reflecting population substructure or genetic ancestry were generated using EIGENSTRAT [18].

Proteomics measurement Proteins were measured in plasma collected at visit 3 (1993–1995) using a modified DNA aptamer-based array that measures 4870 proteins (SomaScan version 4.0, SomaLogic, USA), described previously [19, 20].

Samples that had been stored at -70°C and not previously thawed were transferred to the SomaLogic laboratory and incubated with proprietary reagents. Protein levels were quantified using single-stranded DNA-based modified aptamers that bind to specific protein epitopes. Protein concentrations are reported as relative fluorescent units.

Proteomics quality control Protein measurements by SomaScan were standardised and normalised as previously described [21, 22]. Briefly, hybridisation control normalisation was applied to each sample based on a set of hybridisation control sequences to correct for systematic biases during hybridisation. Median signal normalisation was applied to measures within plates to remove sample or assay biases due to variations in pipetting, reagent concentrations, assay timing and other sources of systematic variability within single plate runs. Each plate contained calibrators for each aptamer reagent to correct for plate-to-plate variation based on global reference materials. ARIC investigators previously conducted a pilot study of SomaScan version 3 in 42 ARIC participants and reported excellent metrics of assay reproducibility: median coefficient of variance (Q1–Q3) of 5.0 (4.1–6.9) and median intraclass correlation (Q1–Q3) of 0.96 (0.92–0.98) [22].

Logarithmic transformations (base 2) were applied to all proteins prior to analysis. A total of 422 blind duplicate plasma aliquots were included, and the median inter-assay Bland–Altman coefficient of variation was 6.3%. The median split sample reliability coefficient was 0.85 after applying the following quality control filters on 5284 available aptamer measurements: Bland–Altman coefficient of variation $>50\%$ or a variance of <0.01 on the log scale ($n=94$ excluded) and non-specific binding to non-proteins ($n=313$ excluded). After all quality control measures were completed, 4870 aptamer measurements remained, corresponding to 4697 unique human proteins or protein complexes.

Assessment of kidney function The eGFR at visit 3 was included as a covariate to account for the influences of kidney function on plasma proteins. The Chronic Kidney Disease Epidemiology Collaboration combined creatinine–cystatin C equation was used to estimate GFR (ml/min per 1.73 m^2) [23]. A creatinase enzymatic method using a Roche Modular P Chemistry Analyzer measured serum creatinine (Roche Diagnostics, USA) and was standardised to isotope-dilution mass spectrometry as described previously [24]. A turbidimetric immunoassay (Gentian, Norway) calibrated and standardised to the International Federation of Clinical Chemistry and Laboratory Medicine reference was used to measure serum cystatin C level [25].

Diabetes ascertainment Prevalent diabetes at visit 3 was defined by a fasting blood glucose level ≥ 7.0 mmol/l, non-fasting blood glucose ≥ 11.1 mmol/l, self-reported physician diagnosis of diabetes, or self-reported use of diabetes medications. Among those without prevalent diabetes at visit 3, incident diabetes was obtained by self-reported physician diagnosis of diabetes or self-reported use of diabetes medications at visit 4 (between 1996 and 1998) and in annual or semi-annual follow-up telephone interviews between 1999 and 2019.

Statistical analyses A total of 12,887 participants attended visit 3. After excluding those with missing genetic data ($n=2737$), proteomic data ($n=1041$) or covariate data ($n=194$), 1674 Black and 7241 White participants were available for the main analysis of GRS–protein associations. Race-specific linear regression models were used to estimate associations between each of the six GRSs and proteins. Covariate adjustments were made for age, sex, field centre, eGFR at visit 3, and ten principal components of ancestry (unadjusted analyses shown in electronic supplementary material [ESM] Table 1). For each protein, participants with values that were more than 6 SD from the population mean were excluded. Bonferroni correction was applied to account for multiple testing: the 4870 protein outcomes and six GRSs stipulated a significance threshold of $p < 1.71 \times 10^{-6}$ in White participants. Analyses in Black participants were treated as a replication. Significant replication was defined as having the same direction of association observed in White participants and meeting a Bonferroni-corrected significance threshold that accounts for the number of proteins tested for each GRS in Black participants.

Proteins identified in the main GRS–protein analysis were tested for associations with prevalent and incident diabetes in a secondary analysis, and a schematic of analytical sample sizes is shown in ESM Fig. 1. Logistic regression was used to estimate ORs for each plasma protein with prevalent diabetes at visit 3 among those with all covariate data ($n=11,063$ participants and 1733 cases), with adjustment for age, sex, field centre/race, physical activity based on the ARIC physical activity sport and exercise index [26], BMI, cigarette smoking status and eGFR (all assessed at the visit 3 time point). Field centre and race variables were combined as only Black participants were recruited at the field centre in Jackson, MS. A sensitivity analysis was conducted in which the definition of prevalent type 2 diabetes was limited to self-reported physician diagnosis or use of diabetes medications ($n=10,995$ participants and 1067 cases), since including laboratory values of fasting glucose may inflate the type I error rate. For prospective analysis, Cox regression was used to estimate HRs for incident diabetes over a median follow-up of 23.9 years ($n=6224$ participants, 2354 incident cases) with the same adjustments described above. Date of diagnosis was defined as the date of the interview at

which diabetes was first reported; individuals who remained diabetes-free were censored at the time of their last available interview. For both cross-sectional and prospective analyses, Bonferroni correction was applied to correct for multiple testing: 22 proteins were tested, stipulating a significance threshold of $p \leq 2.3 \times 10^{-3}$. Unadjusted protein–type 2 diabetes analyses shown in ESM Table 2.

Two-sample MR was performed using the MR-Base web application [27] and was restricted to proteins found to be related to both prevalent and incident type 2 diabetes in the secondary analysis. Instrumental variables consisted of protein quantitative trait loci (pQTLs) identified in White ARIC participants by GWAS analyses between the proteins and genetic dosages imputed based on the TOPMed (freeze 5b) reference, with adjustment for age, sex, field centre and ten principal components of ancestry. Clumping was used to prune pQTLs that did not reach the significance threshold ($p \leq 5 \times 10^{-8}$), were in linkage disequilibrium (r^2 for linkage disequilibrium < 0.2) or were within a clumping distance threshold of 10,000 kb. Identified pQTLs in the ARIC study were confirmed using published data from the INTERVAL [28] and AGES-Reykjavik [29] studies. For identified pQTLs, type 2 diabetes summary statistics were obtained from a large consortium study available through the MR-Base platform [12]; this study was selected based on the numbers of type 2 diabetes cases (n for events = 62,892) and overlap with the TOPMed imputation panel used in the ARIC study. For pQTLs that were unavailable in the outcome summary data, proxy SNPs in tight linkage disequilibrium ($r^2 \geq 0.8$) were substituted. For proteins with only one pQTL, Wald ratios were calculated. Two and three pQTLs were identified for neurofascin and complement C2, respectively; inverse variance weighted (IVW) meta-analyses of Wald ratios were performed, and an MR-Egger sensitivity analysis [30] was conducted for C2. For significant two-sample MR results, reverse causality was evaluated. Type 2 diabetes-related SNPs were derived from the above consortium study [12]. Type 2 diabetes-related SNPs from this study were tested for their associations with neuropilin-2 and complement C2, and their associations with these proteins were obtained from GWAS by Sun et al [28] and Suhre et al [31], respectively. MR-Egger and IVW estimates were generated.

Canonical pathway enrichment was examined using the Ingenuity Pathway Analysis platform (Qiagen, USA) to determine potential mechanisms and signalling cascades through which GRSs may influence respective pathogenic phenotypes [32]. Entrez Gene IDs, log expression ratios (i.e. variant–protein association estimates) and false discovery rate q values from primary analyses were uploaded. To control for multiple comparisons, a Benjamini–Hochberg false discovery rate was applied and proteins with q values ≤ 0.05 were deemed significant. Core analysis was performed, and direct and indirect experimentally confirmed relationships across species were evaluated.

Results

The analytical sample comprised 8915 participants with a mean age of 60.1 years, of whom 54.1% were female. To assess the potential for selection bias, all 12,887 participants who attended visit 3 were compared to the analytical sample with complete genotype, proteomic and covariate data (Table 1). The analytical sample had a smaller proportion of Black participants than the visit 3 cohort; however, demographic, clinical and lifestyle characteristics were otherwise similar. Upon stratification into Black ($n=1674$) and White ($n=7241$) participant groups, it was found that, compared with White participants, Black participants showed higher proportions of current smokers (17.2% vs 21.7%) and never smokers (38.5% vs 45.4%), and a greater prevalence of type 2 diabetes (12.7% vs 27.7%).

Identification of GRS-related proteins As shown in Fig. 1, the 12-variant type 2 diabetes GRS defining the BMI/lipid phenotype was associated with 14 plasma proteins in White participants. Of these, eight were replicated in Black participants: leucine-rich repeat neuronal protein 1 ($p=2.1 \times 10^{-14}$), MOB-like protein phocein ($p=9.1 \times 10^{-6}$), dehydrogenase/reductase SDR family member 9 ($p=9.0 \times 10^{-7}$), ADP-ribosylation factor-like protein 2 ($p=1.2 \times 10^{-4}$), D-3-phosphoglycerate dehydrogenase ($p=1.5 \times 10^{-13}$), neurofilament light

polypeptide ($p=4.3 \times 10^{-14}$), tubulin-specific chaperone A ($p=9.2 \times 10^{-15}$) and protein S100-A13 ($p=1.0 \times 10^{-13}$).

As shown in ESM Table 3, the 29-variant type 2 diabetes GRS defining the insulin resistance phenotype was related to plasma chymotrypsinogen B2 ($p=1.1 \times 10^{-12}$) and SLIT and NTRK-like protein 3 ($p=1.2 \times 10^{-6}$) in White participants; the latter result was replicated in Black participants ($p=0.02$). The 12-variant type 2 diabetes GRS defining the lipodystrophy phenotype was related to plasma neuromedin-B ($p=7.7 \times 10^{-7}$) in White participants but this result was not replicated in Black participants ($p>0.05$). The 50-variant type 2 diabetes GRS defining the beta cell function phenotype was related to cartilage intermediate layer protein 2 in White participants ($p=2.2 \times 10^{-10}$), but the association was not replicated in Black participants.

Over 300 GWAS-identified gene variants were previously associated with type 2 diabetes but remain uncategorised with respect to a distinct type 2 diabetes phenotype. When we combined these 339 variants into an ‘uncategorised’ phenotype GRS, one significant plasma protein association was observed in White participants: lactase-phlorizin hydrolase ($p=8.5 \times 10^{-27}$); this result was replicated in Black participants ($p=0.02$). Finally, combining all GWAS-derived variants generated a 442-variant GRS that was related to seven proteins in White participants, as shown in Fig. 2. Of these, no associations were replicated in Black participants

Table 1 Demographic, lifestyle and health characteristics of all ARIC study participants at visit 3 compared to those with available proteomic, genetic and covariate data for the main GRS–protein analysis and further stratified by race

| Characteristic | All visit 3 participants ($n=12,887$) | Analytic sample ($n=8915$) | Black participants ($n=1674$) | White participants ($n=7241$) |
|-----------------------------|--|---------------------------------|------------------------------------|------------------------------------|
| Age | 60.0 ± 5.7 | 60.1 ± 5.7 | 59.0 ± 5.7 | 60.3 ± 5.7 |
| Female | 7170 (55.6) | 4824 (54.1) | 1041 (62.2) | 3783 (52.2) |
| Race | | | | |
| Black | 2997 (23.3) | 1674 (18.8) | – | – |
| White | 9852 (76.5) | 7241 (81.2) | – | – |
| Smoking status ^a | | | | |
| Current | 2283 (17.8) | 1607 (18.1) | 359 (21.6) | 1248 (17.2) |
| Former | 5286 (41.1) | 3751 (42.2) | 545 (32.9) | 3206 (44.3) |
| Non-smoker | 5277 (41.1) | 3537 (39.8) | 752 (45.5) | 2785 (37.5) |
| BMI (kg/m ²) | 28.5 ± 5.6 | 28.5 ± 5.5 | 30.5 ± 6.5 | 28.0 ± 5.2 |
| Waist/hip ratio | 0.94 ± 0.07 | 0.94 ± 0.07 | 0.94 ± 0.07 | 0.94 ± 0.08 |
| Fasting glucose (mmol/l) | 6.18 ± 2.36 | 6.17 ± 2.32 | 7.04 ± 3.60 | 5.97 ± 1.85 |
| Total cholesterol (mmol/l) | 5.37 ± 0.98 | 5.37 ± 0.97 | 5.37 ± 1.01 | 5.37 ± 0.96 |
| LDL-cholesterol (mmol/l) | 3.29 ± 0.89 | 3.30 ± 0.89 | 3.34 ± 0.95 | 3.29 ± 0.87 |
| HDL-cholesterol (mmol/l) | 1.35 ± 0.47 | 1.34 ± 0.47 | 1.43 ± 0.48 | 1.32 ± 0.46 |
| Triglycerides (mmol/l) | 1.37 (0.98–1.94) | 1.39 (0.99–1.96) | 1.14 (0.89–1.58) | 1.45 (1.05–2.04) |
| Prevalent diabetes | 1988 (15.5) | 1378 (15.5) | 460 (27.7) | 918 (12.7) |

Values are means ± SD, n (%) or median (Q1–Q3)

^a There are 39 missing and two unknown participant smoking statuses in the visit 3 cohort

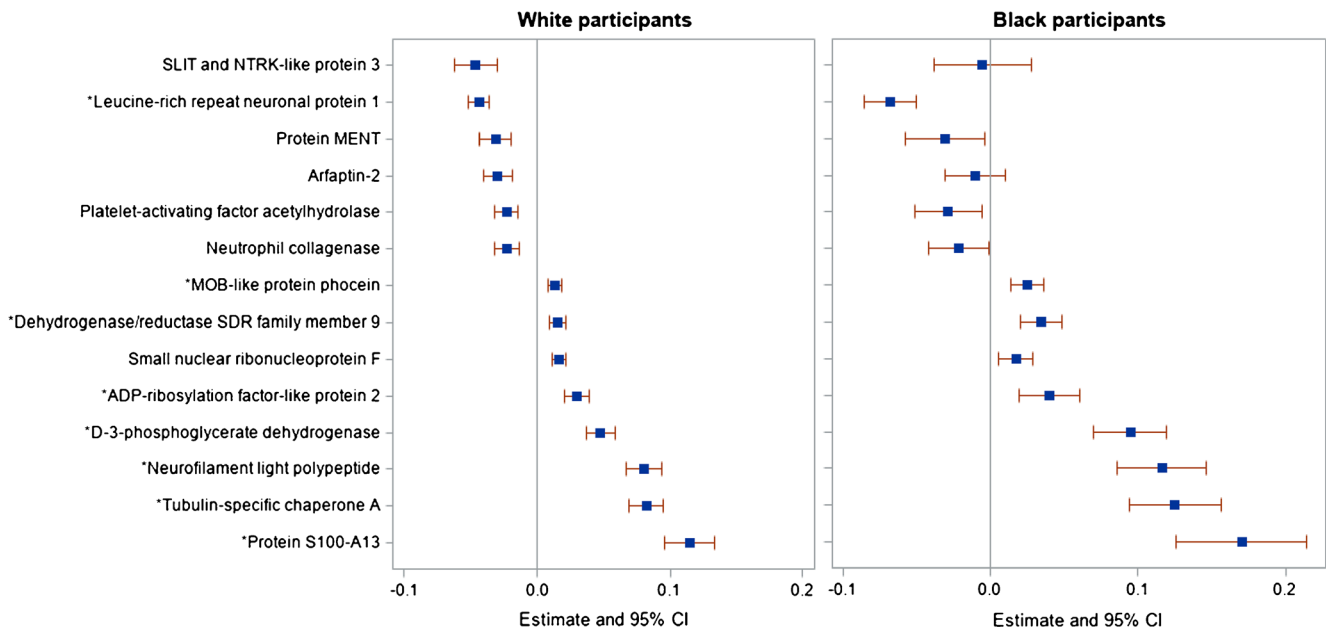


Fig. 1 Plasma proteins that were significantly associated with the type 2 diabetes GRS defining the BMI/lipid phenotype in 7241 White participants, with replication in 1674 Black participants. Results for a regression model examining the BMI GRS and protein outcomes (effect size expressed as difference in log₂ protein level per SD in the GRS), adjusted for age, sex, field centre, ten principal components of ancestry and eGFR

at ARIC study visit 3. The Bonferroni correction for multiple testing was applied with a significance threshold of $p < 1.7 \times 10^{-6}$ in White participants; Black individuals were treated as a replication cohort with a significance threshold of $p < 4.2 \times 10^{-3}$ for correction of 12 tests. Asterisks indicate replication in Black participants

following Bonferroni correction ($p > 7.0 \times 10^{-3}$), but all were directionally consistent. Point estimates, 95% CIs and p values for these associations are provided in ESM Table 3. Associations between GRS exposure variables and metabolic characteristics are shown in ESM Table 4.

A sensitivity analysis was performed in which 555 participants with physician-diagnosed diabetes or those taking diabetes medications were excluded from the GRS–protein analysis; the results are shown in ESM Table 5. Most associations in the subset without diagnosed diabetes were similar in magnitude to those observed in the full analytic sample.

Associations of identified proteins and type 2 diabetes In the secondary analysis, the 22 proteins identified in the main analysis were tested for associations with prevalent and incident diabetes. Among 11,063 Black and White participants at visit 3, there were 1733 individuals with type 2 diabetes. As shown in Fig. 3a, cross-sectional analysis showed that 18 of the 22 proteins were significantly related to prevalent diabetes ($p \leq 2.2 \times 10^{-3}$). Four of these 22 proteins were moderately to strongly correlated with one another. Specifically, neurofilament light polypeptide was correlated with tubulin-specific chaperone protein A ($r = 0.79$; $p < 0.001$), D-3-

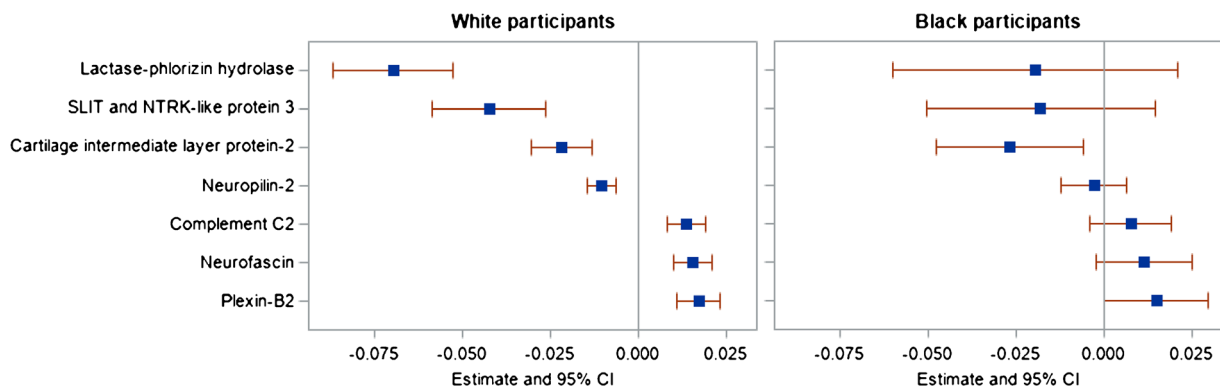


Fig. 2 Plasma proteins that were significantly associated with the overall type 2 diabetes genetic risk score in 7241 White participants, with replication in 1674 Black participants. Results for a regression model examining the GRS of all diabetes-related variants and protein outcomes (effect size expressed as difference in log₂ protein level per SD in the GRS),

adjusted for age, sex, field centre, ten principal components of ancestry, and eGFR at ARIC study visit 3. The Bonferroni correction for multiple testing was applied with a significance threshold of $p < 1.7 \times 10^{-6}$ in White participants; Black individuals were treated as a replication cohort with a significance threshold of $p < 7.1 \times 10^{-3}$ for correction of seven tests

phosphoglycerate-dehydrogenase ($r=0.69$; $p<0.001$) and protein-S100-A13 ($r=0.65$; $p<0.001$). Despite these correlations, all proteins were tested for their associations with type 2 diabetes outcomes.

A sensitivity analysis was performed in which prevalent type 2 diabetes was defined as self-reported physician diagnosis or use of type 2 diabetes medications (n for cases=1069). The results are shown in ESM Table 6 and are consistent with the previous model that used a more expansive definition of type 2 diabetes that included laboratory values of fasting glucose obtained at visit 3, indicating robust associations.

As shown in Fig. 3b, 10 of the 22 plasma proteins were significantly related to incident diabetes over a median follow-up of 23.9 years among 6224 Black and White participants without diabetes at visit 3 ($n=2354$ incident cases). ESM Table 7 provides point estimates, 95% CIs and p values for cross-sectional and prospective analyses.

ESM Table 8 provides a summary of our main findings: protein-GRS associations (0 or 1), replication in Black participants, and protein associations with prevalent and incident type 2 diabetes.

Two-sample MR analysis For the ten proteins associated with both prevalent and incident type 2 diabetes, we performed a two-sample MR analysis to examine relationships between genetically determined protein levels and type 2 diabetes outcomes. Five of the ten proteins were found to have available genetic instruments. The results of this analysis are

presented in Table 2, showing the target protein, corresponding pQTL(s) and r^2 value specifying the percentage variance in protein level explained by the pQTL(s) in the ARIC study. A significant and inverse association was observed for neuropilin-2, suggesting that low neuropilin-2 levels promote type 2 diabetes development (OR per SD 0.44; $p=0.008$). Complement C2 was found to be positively related to type 2 diabetes (OR per SD 1.65; $p=0.007$); however, the MR-Egger results for complement C2 were not significant (OR per SD 1.07; $p=0.96$) and the test for directional horizontal pleiotropy was null ($p=0.72$). Further details of pQTLs are shown in ESM Table 9.

The potential for reverse causality was evaluated for neuropilin-2 and complement C2 (ESM Table 10). For neuropilin-2, 112 type 2 diabetes-related SNPs were available for analysis; estimates were null for IVW analyses (OR per SD 0.87; $p=0.13$) and MR-Egger sensitivity analyses (OR per SD 1.02; $p=0.6$) (ESM Fig. 2). For complement C2, 57 type 2 diabetes-related SNPs were available for analysis; estimates were null for IVW analyses (OR per SD 1.06; $p=0.76$) and MR-Egger sensitivity analyses (OR per SD 1.00; $p=0.96$) (ESM Fig. 3). Tests for horizontal pleiotropy were null for both proteins but approached significance for neuropilin-2 ($p=0.054$).

Pathway enrichment analysis Pathway analysis was performed for proteins associated with each GRS. One canonical pathway was identified in which protein associations with the

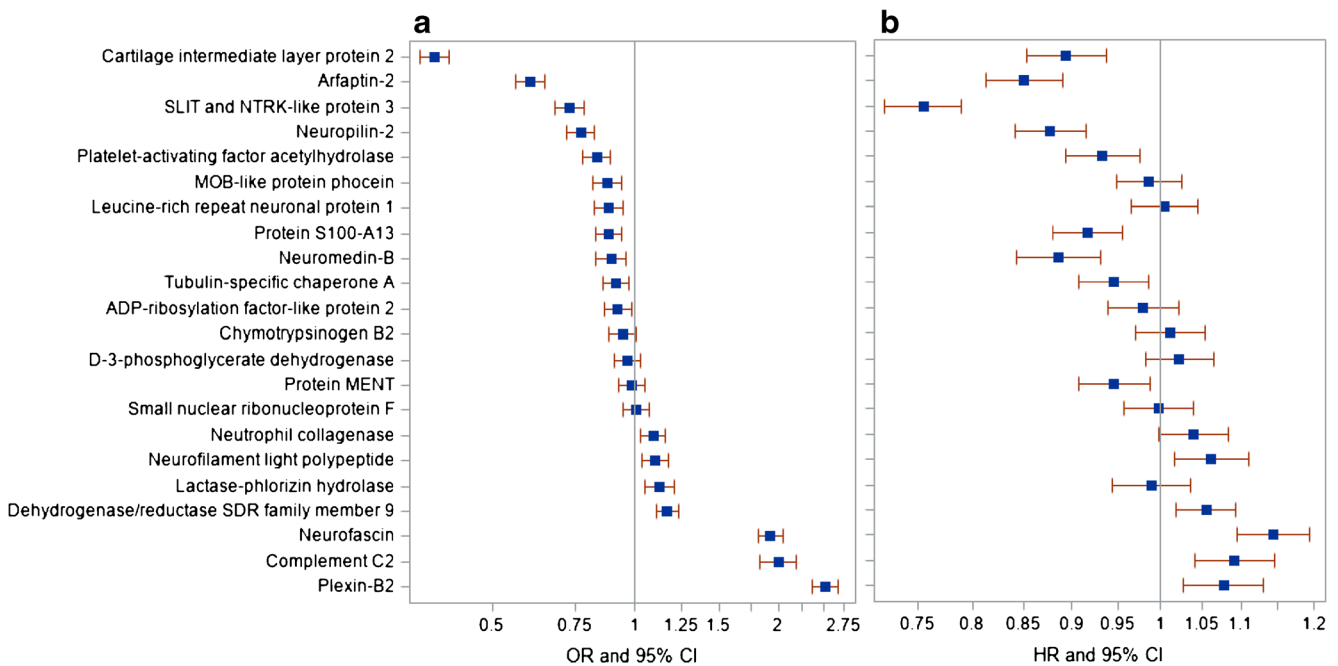


Fig. 3 Associations between identified proteins and (a) prevalent type 2 diabetes, among 11,063 participants at visit 3 (OR and 95% CI are shown; n for events=1733), or (b) incident type 2 diabetes, among 6224 ARIC participants over a median of 23.9 years of follow-up (HR ratios and 95%

CI are shown; $n=2354$ events). Models were adjusted for age, sex, field centre/race, physical activity, BMI, cigarette smoking status and eGFR at visit 3

Table 2 GWAS-identified protein quantitative trait loci and results of two-sample MR analysis

| Protein exposure | pQTL in the ARIC Study | | | | | | | | Two-sample MR results (per SD) ^a | | | |
|-----------------------|------------------------|-----|-----------|-----|-----|------------------------------|-----------------|--------------------|---|----------|----------|----------------------|
| | rsID | Chr | Position | Ref | Alt | <i>cis</i> / <i>trans</i> | Gene | R^2 ^b | OR | Lower CI | Upper CI | <i>p</i> |
| NRP2 | rs16837641 | 2 | 205173653 | T | C | <i>cis</i> | <i>NRP2</i> | 0.006 | 0.44 | 0.24 | 0.8 | 8.0×10^{-3} |
| SLITRK3 | rs11928265 | 3 | 165193012 | A | G | <i>cis</i> | <i>SLITRK3</i> | 0.038 | 0.98 | 0.9 | 1.08 | 0.71 |
| NMB | rs35127183 | 15 | 84672508 | G | A | <i>trans</i> | <i>SEC11A</i> | 0.007 | 0.97 | 0.51 | 1.84 | 0.93 |
| NFASC | rs11801063 | 1 | 204840015 | C | T | <i>cis</i> | <i>NFASC</i> | 0.056 | 1.61 | 1.15 | 2.28 | 6.3×10^{-3} |
| | rs6667532 | 1 | 204979531 | G | A | <i>cis</i> | <i>NFASC</i> | | 1.07 | 0.87 | 1.32 | 0.52 |
| IVW estimate | | | | | | | | | 1.20 | 0.84 | 1.72 | 0.32 |
| C2 | rs558702 | 6 | 31902549 | A | G | <i>cis</i> | <i>C2</i> | 0.02 | 2.46 | 1.45 | 4.18 | 8.7×10^{-4} |
| | rs3130573 | 6 | 31138491 | G | A | <i>cis</i> ^c | <i>PSORSIC1</i> | | 1.66 | 1.05 | 2.62 | 0.03 |
| | rs6916921 | 6 | 31552649 | T | C | <i>cis</i> ^c | <i>NFKBIL1</i> | | 1.26 | 0.82 | 1.95 | 0.30 |
| IVW estimate | | | | | | | | | 1.65 | 1.15 | 2.38 | 7.0×10^{-3} |
| MR-Egger ^d | | | | | | | | | 1.07 | 0.16 | 7.12 | 0.96 |

^aWald ratios are shown for single instrumental variables

^b R^2 values indicate the percentage variance in protein levels explained by the corresponding pQTL (or all loci for C2 and NFASC) in the ARIC Study

^crs3130573 and rs6916921 are within 1 Mb of the *C2* gene and are classified as *cis*-pQTLs

^dEgger intercept=0.019

NRP2, neuropilin-2; SLITRK3, SLIT and NTRK-like protein 3; NMB, neuromedin-B; NFASC, neurofascin; C2, complement C2

GRS defining the BMI/lipid phenotype and plasma proteins indicated retinoate biosynthesis I activation (z score 2.24; $p=2.3 \times 10^{-7}$). Pathway components included alcohol dehydrogenase 4 (q value=0.03), aldo-keto reductase family 1 member C4 (q 0.02), dehydrogenase/reductase SDR family member 9 (q 0.0002) and retinol binding protein 5 (q 0.03).

Discussion

Proteomics research has examined associations with prevalent and incident T2D, its complications and responses to pharmacotherapies, but no proteomics study has examined the genetic liabilities captured by previously generated GRS specific for T2D phenotypes. Using a large-scale agnostic proteomics approach in White participants, we identified 22 unique plasma proteins associated with GRSs for BMI, insulin resistance, beta cell dysfunction and lipodystrophy, or a composite of all type 2 diabetes-related variants. Of these, ten proteins were replicated in 1674 Black participants, and 21 of the 22 associations were directionally consistent between the discovery and replication samples. Of these 22 proteins, 18 were statistically significantly related to prevalent type 2 diabetes, and ten of these were related to incident diabetes over a median of 24 years of follow-up. MR analysis showed that higher neuropilin-2 levels may be protective against type 2 diabetes, while higher complement C2 levels may be related to disease development based on the IVW estimate, although this was tempered by the MR-Egger result.

Collectively, these findings suggest potential mechanisms through which the phenotype-specific GRSs confer greater risks of type 2 diabetes.

Of the identified proteins, plexin-B2, S100-A13 arfaptin-2, neurofascin and complement C2 have previously been shown to be associated with prevalent and/or incident type 2 diabetes [33]. The plexin-B2 receptor and its ligand, semaphorin 6A (with a significance level of $p=1.1 \times 10^{-5}$ in the fully adjusted model, above the Bonferroni-corrected threshold), have been shown to be associated with HbA_{1c} levels in individuals with type 2 diabetes [34], and circulating levels of plexin-B2 have been shown to be positively related to greater risk of prevalent type 2 diabetes, but not incident type 2 diabetes [33]. In contrast, protein S100-A13 has been suggested to have a protective effect against adverse metabolic outcomes [33, 35]. As shown here and by Gudmundsdottir et al in the AGES-Reykjavik cohort [33], lower levels of protein S100-A13 were related to both prevalent and incident type 2 diabetes. In agreement with these findings, hypomethylation of the *S100A13* gene has been shown to be associated with lower protein levels and prevalent diabetic retinopathy, potentially through NF- κ B activation and greater hyperglycaemia-induced vascular damage [35]. We were unable to identify a suitable genetic instrument for these proteins for evaluation in MR analysis.

Apart from the above five proteins, the remaining identified proteins are novel observations, and few have been characterised in type 2 diabetes development—indeed, most have functions

that are seemingly unrelated to metabolic health or disease. Leucine-rich repeat neuronal protein 1, neuromedin-B, neurofilament light polypeptide and neuropilin-2 have been characterised in gastric cancer [36], smooth muscle contraction [37], head and neck cancer [38] and angiogenesis [39], respectively. Despite this, we report here that the semaphorin receptor, neuropilin-2, may have a protective role against disease development based on MR results.

Arfaptin-2 and neurofascin do not have any known physiological roles in metabolic function or adipogenesis despite their associations with type 2 diabetes shown here. However, consistent with a potential protective effect, the ubiquitously expressed arfaptin-2 protein has been shown to inhibit NF- κ B activity [40]. Whether arfaptin-2 suppression of NF- κ B signalling influences adiposity or predisposition to adipose tissue accumulation has not been shown, but a central role for NF- κ B has been reported in models of obesity [41] and insulin resistance [42].

Finally, we confirm that plasma levels of complement C2 are positively associated with risk of type 2 diabetes as shown previously [33], and the results of the MR analysis suggest a causal relationship. These findings are consistent with the established role of the complement system in inflammation and its deleterious effects on metabolic function. Recent studies have shown that activation of complement components likely promote insulin resistance [43, 44] and microvascular complications including nephropathy [45–47] and retinopathy [48, 49]. However, the IVW result must be considered in combination with the non-significant result of the MR-Egger sensitivity analysis. Based on the latter finding and the null directional horizontal pleiotropy finding, it appears that the instrumental variable for complement C2 likely violated the ‘instrument strength independent of direct effect’ assumption [50]. Critically, of the three pQTL components of the C2 instrumental variable, the pQTL proximal to the C2 gene (rs558702) is an intron variant, showed the largest magnitude of association with type 2 diabetes (OR per SD 2.46; 95% CI 1.45–4.18) and is more likely to be independent of horizontal pleiotropy. It may therefore provide a more reliable estimate of the effect of complement C2 on type 2 diabetes. Taken together, the potential causal effect of complement C2 on type 2 diabetes risk remains equivocal. Additional research is warranted to further characterise the role of C2 and other complement system components in type 2 diabetes.

An important consideration in evaluating the present findings is the degree of pathway convergence, which depends on the variant composition of the GRS. It has been suggested that a GRS may capture convergence in downstream disease pathways that originate with hundreds to thousands of genetic risk variants [33]. In that case, a GRS comprised of variants that operate through a shared pathway(s) would be expected to show moderate to strong associations with protein components of that pathway. Conversely, a GRS comprised of

variants that operate through different pathways would be expected to show more modest associations with proteins specific to one pathway. The relatively small magnitudes of association observed here suggest that the latter is occurring, and a few gene variants within each GRS may be driving the significant findings. In addition, these GRSs are likely incomplete as not all variants contributing to a specific type 2 diabetes phenotype are currently known. Taken together, these observations may have implications for future studies to: (1) sub-classify known variants by shared pathways; and (2) identify new variants and epigenetic modifications to obtain a more complete representation of genetic liability, which may then inform the degree of pathway convergence for each diabetes phenotype.

Limitations There are limitations in the present analysis that must be acknowledged. First, GRS construction assumed an additive genetic architecture of independent risk variants and did not account for the possibility of gene–gene or gene–environment interactions. Additionally, gold standard assessments of beta cell function, insulin resistance and lipodystrophy were not available in ARIC participants, and we were unable to assess relationships between identified proteins and these phenotypic manifestations of type 2 diabetes. Our two-sample MR analysis was limited by two factors: (1) no genetic instruments were found for cartilage intermediate layer protein-2 in the ARIC study; and (2) outcome summary statistics for the s100-A13, arfaptin-2, plexin-B2 and platelet activating factor-acetyl hydrolase pQTLs or identified proxy SNPs were unavailable in the study selected from MR-Base, which prevented MR analysis of these proteins. Limiting the generalisability of our findings, GRSs were comprised of variants identified from GWA studies conducted in individuals of European ancestry, and this may have contributed to the relatively moderate replication of GRS–protein associations among Black participants (ten of 22 proteins); however, the directional consistency in associations between groups was substantial: 21 of the 22 proteins identified in White participants were directionally consistent in Black participants. Finally, we had limited statistical power to identify pQTLs in Black participants and were therefore unable to perform MR analysis in that group; additional studies with larger samples of Black individuals are needed.

Conclusions Using a large-scale agnostic proteomic approach, we identified 22 novel proteins associated with GRS for type 2 diabetes in the ARIC study. Most of these were found to be associated with prevalent or incident type 2 diabetes after adjustment for potential confounders. Two-sample MR analysis provided evidence that complement C2 and neuropilin-2 levels may be causally related to type 2 diabetes risk, although the former finding should be interpreted with

caution. Additional research is warranted to further interrogate these proteins and characterise their possible roles in the pathophysiology of type 2 diabetes and as potential targets for pharmacological intervention.

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Data availability The datasets analysed for this study are available from the ARIC Coordinating Center (arichelp@unc.edu) on reasonable request, which may include completion of a data and material distribution agreement.

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Authors' relationships and activities ES is a member of the Editorial Board of *Diabetologia*. The authors declare that there are no other relationships or activities that might bias, or be perceived to bias, their work.

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