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ORIGINAL RESEARCH - CLINICAL

Proteome-Wide Genetic Investigation of Large Artery Stiffness

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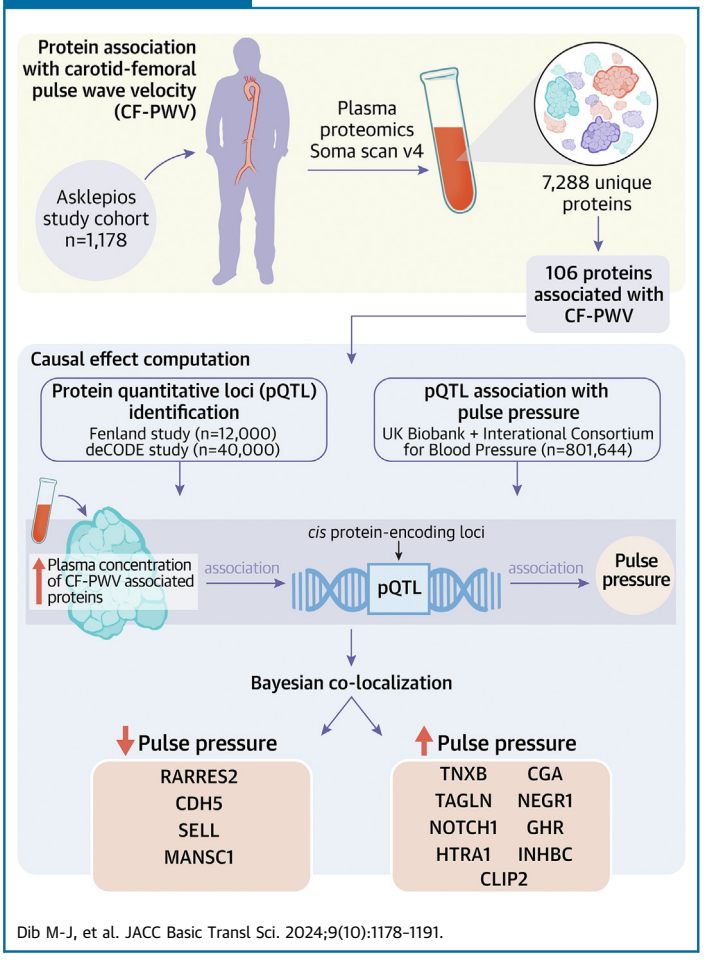
VISUAL ABSTRACT



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HIGHLIGHTS

- **Identification of 106 novel plasma proteins associated with LAS in middle-aged adults.**
- **Prioritization of 13 candidate proteins with a causal effect on pulse pressure.**
- **Targeting LAS beyond traditional risk factors may prevent CVD and target organ damage.**

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SUMMARY

The molecular mechanisms contributing to large artery stiffness (LAS) are not fully understood. The aim of this study was to investigate the association between circulating plasma proteins and LAS using complementary proteomic and genomic analyses. A total of 106 proteins associated with carotid-femoral pulse-wave velocity, a noninvasive measure of LAS, were identified in 1,178 individuals from the Asklepios study cohort. Mendelian randomization analyses revealed causal effects of 13 genetically predicted plasma proteins on pulse pressure, including cartilage intermediate layer protein-2, high-temperature requirement A serine peptidase-1, and neuronal growth factor-1. These findings suggest potential novel therapeutic targets to reduce LAS and its related diseases. (JACC Basic Transl Sci. 2024;9:1178–1191) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ABBREVIATIONS AND ACRONYMS

CDH5 = cadherin-5 4

CF-PWV = carotid-femoral pulse-wave velocity >20

CILP2 = cartilage intermediate layer protein-2 5

COL1A1 = collagen 1 alpha-1 chain 4

FABP = fatty acid binding protein 12

HTRA1 = high-temperature requirement A serine peptidase 1 10

INHBC = inhibin beta C chain 3

LAS = large artery stiffness >20

MANSC1 = MANSC domain-containing protein-1 3

MR = Mendelian randomization >20

NEGR1 = neuronal growth regulator-1 3

NOTCH1 = neurogenic locus notch homolog protein 1 4

PP = pulse pressure >20

RARRES2 = retinoic acid receptor responder protein-2

TAGLN = transgelin 8

VE = vascular endothelial 8

Large artery (aortic) stiffness (LAS) and subsequent adverse pulsatile hemodynamic changes are manifestations of vascular aging that play a central role in the pathogenesis of various cardiovascular diseases.[1-3] LAS increases markedly with age because of structural changes in the medial layer of the arterial wall and is accelerated by various cardiovascular risk factors, such as diabetes and hypertension.[4] LAS has multiple adverse hemodynamic consequences, including increased left ventricular afterload, and increased pulse pressure (PP), leading to excess pulsatility penetration into the microvasculature of various target organs. Of note, LAS is not pathologically similar to atherosclerosis; rather, it occurs primarily in the medial arterial layer and can occur in the absence of plaque formation.[5] Therapeutically targeting LAS is therefore desirable for the improvement of the global burden of cardiovascular disease.[5] Carotid-femoral pulse-wave velocity (CF-PWV) is considered the reference noninvasive metric of LAS[6] and is independently associated with a greater risk for heart failure-related hospitalization,[7] progression to end-stage kidney disease, all-cause death, and various other adverse cardiovascular events.[6,8] Increased PP, a direct hemodynamic consequence of LAS, is also associated with cardiovascular disease and cardiovascular risk.[9]

Technological advances have recently enabled the assessment of thousands of circulating proteins in relation to adverse health outcomes.[10-15] Two previous studies assessed correlations between a small number of plasma proteins (n = 92) among healthy participants[16] and in patients with chronic kidney disease[17] and LAS. However, given the small samples sizes and limited number of measured proteins, only a few associations were reported. Moreover, the causality of these associations was not directly investigated.

Genome-wide association of plasma proteomics, applied among large population cohorts,[18-20] has

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paved the way for enhanced insights into the genetic regulation of the plasma proteome, providing a unique opportunity to evaluate the causal effects of plasma proteins on the development and progression of various conditions. Mendelian randomization (MR) is a powerful method that enables the identification of causal effects in humans, transcending some limitations of traditional observational studies such as residual confounding and reverse causality. MR leverages the naturally randomized allocation of genetic variants among the population as instrumental variables, analogous to treatment allocation in a randomized controlled trial. Under certain assumptions, this approach estimates the causal effects of exposures on outcomes.[21] Given the significant heritability of CF-PWV (11%-40%)[22,23] and PP (24.4%),[24] genetically predicted levels of LAS represent a key opportunity to assess the causal effects of specific proteins within the MR paradigm. To date, no large studies have assessed the putative causal association between the plasma proteome and indexes of LAS, a major determinant of cardiovascular risk.

In this study, we aimed to: 1) evaluate the relationship between plasma proteins and CF-PWV in middle-aged adults from the community-based Asklepios study; and 2) assess the potential causal associations between specific plasma proteins and PP using MR.

METHODS

The data supporting the findings of this study can be made available for collaborative research upon the execution of appropriate data-sharing agreements; please request from E.R.R. (ernst.rietzschel@ugent.be). We first conducted a proteome-wide association study to identify plasma proteins associated with CF-PWV in the Asklepios cohort. Statistically significant associations were taken forward to *cis*-MR analyses, in which the associations of genetically predicted proteins with PP, a surrogate marker of LAS, were assessed.

ASKLEPIOS STUDY. The Asklepios study is a longitudinal study including 2,524 middle-aged individuals (1,301 women and 1,223 men) for the first round of data collection aged 35 to 55 years from 2 Belgian communities. The study has been previously described in detail.[25,26] None of the participants had apparent cardiovascular disease (including valvular heart disease) with echocardiographic measurements at the time of enrollment. The Ghent University hospital ethics committee approved the study protocol, and participants provided written informed consent (approval number 2002/133).

PLASMA PROTEIN QUANTIFICATION. Plasma samples from 1,250 randomly selected Asklepios study participants were collected, split into aliquots, transferred into CryoTube vials (Nunc), and stored on site at −80 °C. More specifically, the EDTA plasma samples analyzed for this study were blinded to the operators, randomly dispersed across 5 plates, and assayed over the course of 2 days. Each plate contained 85 test samples, 5 calibrators, 3 quality-control samples, and 3 buffer replicates. This randomization combined with normalization to an external reference (normal-based U.S. population) ensures systematic bias is minimized. Aliquots of each sample were prepared by the study team and shipped frozen to SomaLogic, where they were thawed, aliquoted for assay, frozen, then thawed again the day of assay. All samples were subjected to 3 freeze-thaw cycles. Equal freeze-thaw cycles across sample pairs and treatment groups further minimize the potential for pre-analytical variability to affect analysis. Standardized sample-handling protocols were used. Plasma samples that were collected at baseline were analyzed using the SomaScan assay version 4.1 (SomaLogic), which is a multiplexed, modified aptamer-based binding assay. The SomaScan assay uses slow-off-rate modified aptamer (SOMAmer) reagents, which are chemically modified nucleotides, to bind and quantify target proteins in relative fluorescent units directly proportional to the amount of target protein in the sample.[27] The SomaScan assay included 7,523 modified aptamer reagents to 7,288 unique protein targets.[28] The SomaScan assay is performed under the SomaLogic Quality System in a laboratory that follows Clinical Laboratory Improvement Amendments standards for laboratory developed tests. SomaScan assay controls and data standardization procedures were performed by SomaLogic according to their protocols and as previously described.[28] This included hybridization normalization to account for variability in the readout of individual microarrays by adding control SOMAmer reagents not exposed to protein to the microarray slide, intraplate median signal normalization of matrix-matched calibrator control replicates to account for technical variability among replicates within a run, plate scaling and calibration to mitigate variation between assay runs and account for batch effects, normalization to a reference using adaptive normalization by maximum likelihood, and quality-control checks to evaluate the accuracy of the assay after data standardization using pooled matrix-matched quality controls run in replicate alongside samples.

Internal testing of the performance characteristics of the SomaScan assay by SomaLogic using triplicates

from 4 healthy plasma samples run across 15 plates revealed a median intraplate coefficient of variation of 3.6%, a median interplate coefficient of variation of 3.8%, and a total coefficient of variation of 5.3% for human plasma samples (www.somalogic.com). In addition, all SOMAmer reagents have been tested for determination of the equilibrium binding affinity dissociation constant, in pull-down assays of cognate protein in buffer using the SOMAmer reagent, for demonstration of buffer dose response in the SomaScan assay, and for estimation of endogenous signal in plasma. Studies carried out by independent laboratories have confirmed consistent assay performance at coefficients of variation at or <5%.[29-34]

CF-PWV MEASUREMENTS. CF-PWV is currently considered the reference noninvasive metric for LAS.[5,6] CF-PWV was measured in 1,178 individuals with SomaScan measurements via pulsed-wave Doppler interrogations of the carotid and femoral arteries using a commercially available ultrasonographic system (Vivid 7, GE-Vingmed Ultrasound) equipped with a vascular transducer (12L, 7.3-11.4 MHz; linear-array transducer set at 10 MHz) in the supine position. The time delay between carotid and femoral sites was computed using the QRS complex as a fiducial point. CF-PWV was computed as the distance between the measurement sites divided by the time delay and expressed in meters per second.

STATISTICAL AND BIOINFORMATICS ANALYSES.

Proteome-wide association study of CF-PWV. We assessed the relationship between levels of circulating proteins and CF-PWV using robust linear regression, after Box-Cox transformation, to improve the normality of the distribution of CF-PWV and analyzed proteins, as appropriate. We corrected the alpha level for multiple comparisons using the number of principal components underlying >95% of the variability of all measured proteins, as previously described.[15,35-37] Statistical significance was defined as a multiplicity-corrected *P* value of <0.05. We also considered a 5% false discovery rate threshold as sensitivity analysis for the correction. All probability values presented are 2-tailed. Proteome-wide associations were used to perform pathway analyses.

PATHWAY ANALYSES. Ingenuity Pathway Analysis software (Qiagen) was used to conduct pathway analyses. Proteins were identified according to their UniProt identifier annotations. The totality of proteins included in the SomaScan assay was used as the reference set, and both direct and indirect experimentally confirmed relationships from all species were included. The Core analysis module in Ingenuity Pathway Analysis was used to perform pathway analysis on the differentially expressed proteins. This analysis identifies specific canonical pathways in which the changes are highlighted. The analysis calculates a *P* value (Fisher exact test and right tailed), quantifying the overlap, and a *z*-score, quantifying the likelihood and direction (up- or down-regulated) between the plasma proteomics pattern and known canonical pathways.

2-sample MR analyses. We conducted 2-sample *cis*-MR analyses to estimate the association of genetically predicted CF-PWV-associated plasma protein levels on PP, a hemodynamic marker directly affected by LAS, which has well-established genetic predictors. MR is a statistical approach that uses genetic variants as instrumental variables to proxy the causal effects of an exposure on an outcome of interest under the relevance, independence, and exclusion restriction assumptions.[21] We used genetic summary statistics from the PP meta-analysis of genome-wide association studies from the UK Biobank Neale Lab and International Consortium for Blood Pressure, which included 801,644 participants.[4] Using proteomics data from the Icelandic deCODE[18] and Fenland studies,[38] we first identified *cis* (within ±500 kb of the protein-encoding region) acting protein quantitative trait loci for circulating protein levels of each of the identified proteins in the discovery phase. Proteins were analyzed if protein quantitative trait loci with association *P* values $<5.0 \times 10^{-8}$ were available. MR estimates were considered statistically significant at a 5% false discovery rate threshold. We report MR estimates as regression coefficients (β) with 95% CIs for the putative effects of these proteins on PP. β values represent the changes in PP (in millimeters of mercury) for every SD change in genetically predicted plasma protein levels. Proteins that were significantly associated with PP were the subject of genetic colocalization analyses.

GENETIC COLOCALIZATION ANALYSES. Upon identifying MR evidence of a causal effect, we conducted genetic colocalization analyses as sensitivity to confirm that the proteins with putative causal effects on PP (as indicated by MR) were regulated by the same causal variant.[39] The colocalization method calculates posterior probabilities for the competing hypotheses of H_0 (no causal variants), H_1 (causal variant for trait 1, the protein level in our case), H_2 (causal variant for trait 2, the PP outcomes in our case), H_3 (distinct causal variants for traits 1 and 2), and H_4 (shared causal variants for traits 1 and 2, which supports a causal association). The prior probability that a single nucleotide polymorphism is causal to 1 trait in a region was set to 10^{-4}. If the posterior

probability that 1 single nucleotide polymorphism is shared for traits 1 and 2 was >0.8, we regarded it as a colocalization signal, supporting a causal effect of the protein on the outcome. Conditional colocalization (posterior probability$_{H4}$ / [pPosterior probability$_{H4}$ + posterior probability$_{H3}$]) was performed to evaluate the evidence supporting a shared causal variant, conditional on the presence of causal variants for each trait in the region. We also conducted sensitivity analyses to consider whether conclusions were robust. We calibrated priors to p_1 (the probability that the single nucleotide polymorphism is associated with protein levels only), p_2 (the probability that the single-nucleotide polymorphism is associated with PP only) and p_{12} (the probability that an arbitrary single nucleotide polymorphism is associated with the trait for both protein levels and PP) at 10^{-4}. To assess for robustness of posterior inference, prior sensitivity was tested by varying p_{12} from 10^{-4} to 10^{-8} and keeping p_1 and p_2 constant at 10^{-4}. The rationale is that we can use such sensitivity analysis to consider whether conclusions are robust over a range of plausible p_{12} values.

SOFTWARE. Analyses were performed using the MATLAB statistics and machine learning toolbox (The MathWorks) and R version 4.0.3 (R Foundation for Statistical Computing). We used the MendelianRandomization (version 0.6.0) and TwoSampleMR (version 0.5.6) packages in R to perform all MR analyses. Colocalization was performed using Coloc (version 3.2-1).

RESULTS

General characteristics of the Asklepios study participants are summarized in **Table 1**.

PROTEOME-WIDE ASSOCIATION STUDY OF CF-PWV. In the Asklepios cohort, we found 106 proteins to be associated with CF-PWV in regression analyses. Volcano plots showing the associations between CF-PWV and other plasma protein levels in both models are shown in **Figure 1A**. β estimates and *P* values for the proteins with statistically significant associations are shown in Supplemental Tables 1 and 2.

The top proteins positively associated with CF-PWV in these analyses included fatty acid binding protein 3 (FABP3), FABP4, carboxylesterase-1, leptin, plasminogen activator, tissue type, sclerostin, fructose-1,6-bisphosphate aldolase, aminoacylase-1, fructose-bisphosphatase-1, and glycerol-3-phosphate dehydrogenase-1. The top proteins negatively associated with CF-PWV included immunoglobulin superfamily DCC subclass member-4; neuronal growth regulator-1 (NEGR1); potassium voltage-gated channel subfamily A member-10; ADP-ribosyltransferase-3; transforming growth factor beta receptor type-3; delta-like protein-4; MANSC domain-containing protein-1 (MANSC1); follistatin/kazal, immunoglobulin, kunitz, and netrin domain containing-2; collagen 1 alpha-1 chain (COL1A1); and secretogranin-3.

The top 5 canonical pathways significantly associated with CF-PWV using proteomic associations from our aforementioned analyses are shown in **Figure 2A**. Various inflammatory and metabolic pathways were prominent processes highlighted in the analysis. They included the granulocyte adhesion and diapedesis pathway, the urea cycle, the farnesoid X receptor/retinoid X receptor activation pathway, lipopolysaccharide/interleukin-1-mediated inhibition of retinoid X receptor function, and maturity-onset diabetes of the young signaling. In addition, neurohormonal pathways related to catecholamine and steroid hormone metabolism (including glucocorticoid and mineralocorticoid synthesis), as well as pathways related to calcification, were significantly associated with CF-PWV.

In sensitivity analyses adjusted for age, we identified 87 proteins associated with CF-PWV. Top proteins in these analyses were consistent with those identified in the aforementioned unadjusted analyses (**Figure 1B**). These analyses also highlighted inflammatory, metabolic, neurohormonal, and calcification pathways. The top canonical pathways significantly associated with CF-PWV in these analyses adjusted included maturity-onset diabetes of the young signaling, farnesoid X receptor/retinoid X receptor activation, atherosclerosis signaling, the activin inhibin signaling pathway, and dendritic cell maturation.

TWO-SAMPLE MR ANALYSES. Among the 106 proteins associated with CF-PWV in the aforementioned analyses, 67 and 48 had known *cis*-protein quantitative trait loci in the deCODE and Fenland data sets, respectively. Forty-three proteins had genetic instruments available from both data sets. Among those, genetically predicted levels of 5 proteins were associated with PP (**Figure 3**): cadherin-5 (CDH5; $\beta_{deCODE} = -0.23$ [95% CI: −0.34 to −0.11], $\beta_{Fenland} = -0.18$ [95% CI: −0.28 to −0.08]), neurogenic locus notch homolog protein 1 (NOTCH1; $\beta_{deCODE} = 0.34$ [95% CI: 0.16-0.52], $\beta_{Fenland} = 0.22$ [95% CI: 0.07-0.43]), retinoic acid receptor responder protein-2 (RARRES2; $\beta_{deCODE} = -0.13$ [95% CI: −0.21 to −0.05], $\beta_{Fenland} = -0.09$ [95% CI: −0.15 to −0.03]), tenascin XB ($\beta_{deCODE} = 0.17$ [95% CI: 0.06-0.28], $\beta_{Fenland} = 0.10$ [95% CI: 0.07-0.13]), and transgelin (TAGLN;

$\beta_{deCODE} = 0.23$ [95% CI: 0.07-0.40], $\beta_{Fenland} = 0.44$ [95% CI: 0.26-0.62]) at 5% false discovery rate threshold significance.

Twenty proteins had genetic instruments available from only the deCODE data set. Among those, genetically predicted levels of 3 proteins were associated with PP (Figure 3): cartilage intermediate layer protein-2 (CILP2; $\beta = 0.45$; 95% CI: 0.26-0.63), MANSC1 ($\beta = -0.11$; 95% CI: −0.18 to −0.04), and inhibin beta C chain (INHBC; $\beta = 0.28$; 95% CI: 0.11-0.45). Finally, 5 proteins had genetic instruments available from only the Fenland data set. Among those, genetically predicted levels of 5 proteins were associated with PP (Figure 3): serine protease high-temperature requirement A serine peptidase 1 (HTRA1; $\beta = 0.20$; 95% CI: 0.08-0.32), CGA ($\beta = 0.24$; 95% CI: 0.09-0.39), NEGR1 ($\beta = 0.14$; 95% CI: 0.04-0.23), SELL ($\beta = -0.04$; 95% CI: −0.07 to −0.01), and GHR ($\beta = 0.15$; 95% CI: 0.04-0.27). Full results are shown in Figure 3 and Supplemental Tables 3 and 4.

GENETIC COLOCALIZATION ANALYSES. We performed colocalization analyses to assess whether the estimates obtained in MR analyses between the plasma proteome and PP are due to a shared causal variant rather than genetic confounding by linkage disequilibrium (Supplemental Table 5). The associations of genetic variants with CDH5, NOTCH1, RARRES2, CILP2, and INHBC colocalized with the genetic associations of PP with posterior probabilities >0.8. Although not meeting our selected colocalization threshold, HTRA1 had suggestive colocalization with a conditional posterior probability ($H_4/[H_3 + H_4]$) of 0.38.

Table 2 shows the biologic function of plasma proteins associated with CF-PWV. Supplemental Table 6 shows similar information with multiple references for the interested reader.

TABLE 1 Basic Clinical and Hemodynamic Parameters of 1,178 Participants of the Asklepios Study

	Female (n = 605, 51.35%)	Male (n = 573, 48.64%)
Age, y	45.6 (45.1-46.1)	45.6 (45.1-46.1)
BMI, kg/m^2	24.4 (24.1-24.7)	26.4 (26.1-26.7)
Systolic BP, mm Hg	123 (122-124)	131 (130-132)
Diastolic BP, mm Hg	77.1 (76.4-77.8)	82.2 (81.4-83)
Pulse pressure, mm Hg	45.3 (44.7-45.9)	48 (47.3-48.6)
Heart rate, beats/min	70.5 (69.7-71.4)	67 (66.2-67.8)
Total cholesterol, mg/dL	212 (209-214)	217 (214-220)
HDL cholesterol, mg/dL	68.8 (67.5-70.2)	54.4 (53.3-55.4)
Triglycerides, mg/dL	83.3 (79.9-86.8)	112.3 (107.5-117.1)
LDL cholesterol, mg/dL	120 (118-123)	134 (131-138)
CF-PWV	6.49 (6.38-6.59)	6.5 (6.39-6.6)
Current smokers	92 (15.21)	136 (23.73)
Past smokers	220 (36.36)	330 (57.59)
Diabetes mellitus	5 (0.83)	16 (2.79)
Hypertension	145 (23.97)	203 (35.43)
Medications		
Beta-blocker	42 (6.94)	31 (5.41)
Angiotensin receptor blocker	11 (1.82)	8 (1.40)
Calcium-channel blocker	4 (0.66)	4 (0.70)
ACE inhibitor	13 (2.15)	14 (2.44)
Lipid-lowering therapy	37 (6.12)	43 (7.50)
Insulin	0 (0.00)	2 (18.18)

Values are mean (95% CI) or n (%).

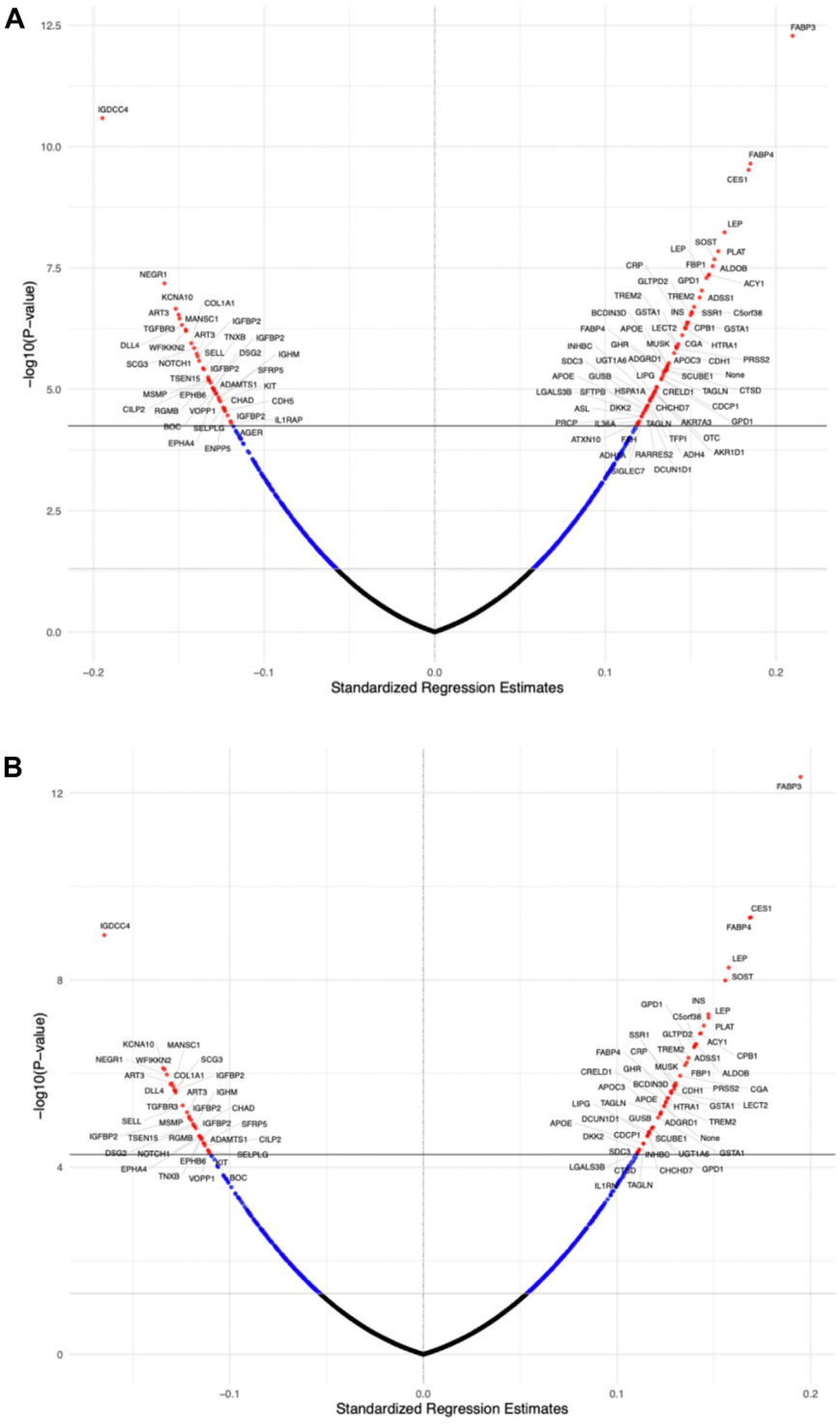
ACE = angiotensin-converting enzyme; BMI = body mass index; BP = blood pressure; CF-PWV = carotid-femoral pulse-wave velocity; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

DISCUSSION

To our knowledge, our study is the first to comprehensively investigate associations between 7,288 proteins and LAS. We leveraged a well-characterized population of 1,178 individuals from the community-based Asklepios study, combined with genomic data from independent cohorts, to gain insights into the putative causal effects of plasma proteins on LAS. We identified a total of 106 proteins that exhibited associations with CF-PWV. MR provided support for causal associations between 13 genetically predicted protein levels and PP, and genetic colocalization validated 6. Our findings identify a large number of novel proteins associated with LAS, validate proteins previously associated with arterial remodeling, prioritize a selected number of novel putative causal proteins, and thus provide preliminary evidence to support future studies of candidate therapeutic targets.

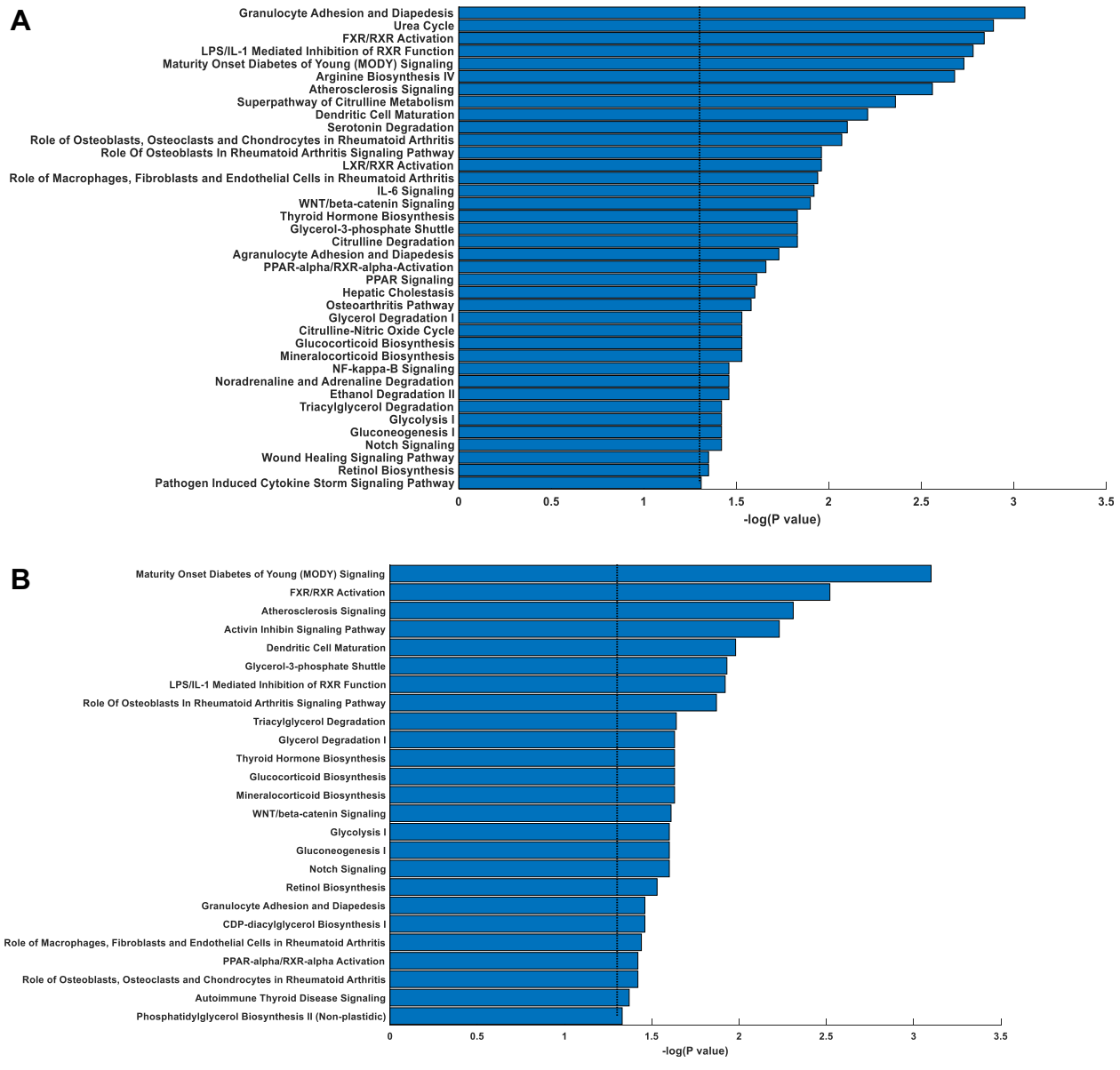
PROTEINS ASSOCIATED WITH CF-PWV. To our knowledge, only 2 previous studies assessed associations between arterial stiffness and multiple plasma proteins. Pettersson-Pablo et al[16] assessed the association of 92 plasma proteins and PWV among 833 healthy adults and identified insulin-like growth factor-binding proteins 1 and 2. Dieden et al[17] assessed associations between 92 plasma proteins and PWV in 362 individuals of a mixed-race study cohort, and FABP4 was associated with PWV in this cohort. Our findings replicate associations with insulin-like growth factor-binding protein-2 and FABP4 from these prior studies. FABP3 and FABP4 were among the top proteins associated with CF-PWV in our study, in both unadjusted and age-adjusted proteome-wide analyses. FABP3, also known as heart-type FABP is a marker of myocardial damage[1] and was found to be associated with increased CF-PWV in patients with newly diagnosed hypertension in a study by Gedikli et al.[40] Despite its name, FABP3 expression is not limited to the heart, and its

FIGURE 1 Associations Between All Plasma Proteins Measured in the Asklepios Study and Carotid-Femoral Pulse Wave Velocity



Volcano plots showing associations between all plasma proteins measured in the Asklepios study and carotid-femoral pulse wave velocity in (A) the unadjusted robust regression model and (B) the model adjusted for age. Multiple instances of the same protein in this plot are due to the presence of multiple aptamers that can bind the same protein in our analyses. Detailed information is available in Supplemental Table 1. These plots show standardized regression estimates against the $\log_{10}$ *P* value. The nominal significance level and the alpha-corrected significance level are represented by dashed lines on the y-axis.

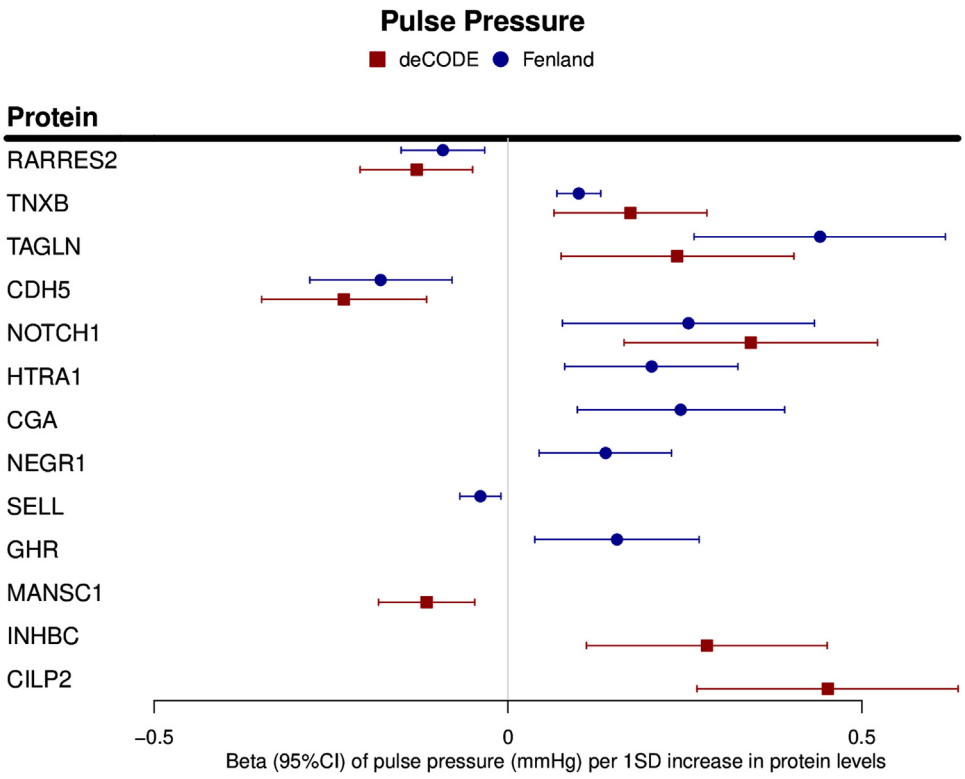
FIGURE 2 **Canonical Pathway Analysis of Proteins Significantly Associated With Carotid-Femoral Pulse-Wave Velocity**



Canonical pathway analysis of proteins significantly associated with carotid-femoral pulse-wave velocity in the robust linear regression model in the Asklepios study in the (A) unadjusted and (B) age-adjusted models. A principal-component analysis–corrected *P* value of 0.05 was used to determine the threshold of significance.

overexpression has been associated with increased extracellular matrix protein expression, increased proliferation of aortic adventitia fibroblasts, and accelerated vascular fibrosis in Takayasu's arteritis.[41] High heart-type FABP levels were also associated with cardiovascular risk factors and were an independent risk factor for cardiovascular death in the longitudinal community-based Takahata study (n = 3,503).[42] FABP4, also known as adipocyte FABP, is expressed mainly in adipocytes and macrophages and is associated with carotid atherosclerosis in individuals with end-stage renal disease and with arterial stiffness in patients with chronic kidney disease.[43] We further report an association between CF-PWV and COL1A1, a fibril-forming collagen found in most connective tissues, including the vascular wall. Abnormal COL1A1 deposition has been suggested to play a role in arterial compliance in genetic

FIGURE 3 Mendelian Randomization Results for the Associations of Genetically Predicted Plasma Protein Levels in the deCODE and Fenland Data Sets With Pulse Pressure



Only significant associations (false discovery rate-corrected $P < 0.05$) for each exposure data set are shown. Mendelian randomization estimates are reported as changes in pulse pressure (millimeters of mercury) per 1-SD change in genetically predicted plasma protein levels.

association studies[44] but has not yet been reported in plasma proteomic studies of arterial stiffness. Various other proteins were identified that may play a role in extracellular matrix remodeling, collagen metabolism, and arterial wall calcification, all of which are relevant biologic processes in LAS.[5] Supplemental Table 6 presents a summary of top proteins associated with CF-PWV and their relevant biologic functions.

Using canonical pathway analysis, we identified several biological pathways associated with CF-PWV, predominantly related to inflammation and metabolism. Inflammation has been previously implicated in LAS in both cross-sectional and experimental studies. Inflammatory mediators may induce LAS through a multitude of mechanisms, including attenuation of endothelial nitric oxide synthase[45] and induction of structural changes in the arterial medial layer.[46] Canonical pathways related to calcification and glucocorticoid and mineralocorticoid metabolism were also found to be associated with CF-PWV in our study. Vascular calcification is a known contributor to LAS.[47] Similarly, mineralocorticoids are known to enhance fibrosis in the aortic wall.[48] The role of these pathways on LAS have been extensively reviewed elsewhere.[49,50]

PROTEINS WITH EVIDENCE OF CAUSAL EFFECTS ON LAS. Our MR analyses with candidate proteins identified in the Asklepios study supported a causal effect of genetically predicted levels of 13 proteins on PP. Whereas some of these proteins have been shown to be involved in arterial remodeling in previous studies (including CDH5, TAGLN, NOTCH1, and HTRA1), most proteins were not previously known to affect PP or LAS.

CDH5 (also known as vascular endothelial [VE]-cadherin) is an adhesion molecule specific to endothelial cells that plays a role in maintaining and controlling their cellular junction. The regulation of VE-cadherin-mediated adhesion mechanisms is key in controlling vascular permeability. VE-cadherin also governs several cellular processes and regulates the function of the VE growth factor receptor. Circulating VE-cadherin levels were found to be elevated in

TABLE 2 Biologic Function of Plasma Proteins Associated With Carotid-Femoral Pulse-Wave Velocity

UniProt ID	Protein	Name	Function
P05413	FABP3	Fatty acid binding protein 3	Differentiation of cardiac myocyte, endothelial function regulation, fatty acid metabolism
Q8TDY8	IGDCC4	Immunoglobulin superfamily DCC subclass member-4	Viral pathogenesis involving influenza virus endocytosis
P15090	FABP4	Fatty acid binding protein 4	Endothelial cell adhesion and inflammation, atherogenesis
P23141	CES1	Liver carboxylesterase-1	Cholesterol regulation, anti-inflammation, bile acid synthesis, lipoprotein metabolism
P41159	LEP	Leptin	Angiogenesis inducer and matrix remodeling, inflammation
P00750	PLAT	Tissue-type plasminogen activator	Fibrinolysis, nitric oxide regulation
Q9BQB4	SOST	Sclerostin	Vascular calcification, Vascular remodeling
P05062	ALDOB	Fructose-bisphosphate aldolase B	Vascular remodeling
Q03154	ACY1	Aminoacylase-1	Collagen regulation and TGF-β1/Smad3 signaling, glucose and insulin homeostasis
P09467	FBP1	Fructose-1,6-bisphosphatase-1	Fibroblast cell regulation, angiogenesis regulation
Q7Z3B1	NEGR1	Neuronal growth regulator-1	A cell adhesion molecule linked to obesity, autism, learning deficits, and increased susceptibility to seizures
P21695	GPD1	Glycerol-3-phosphate dehydrogenase (NAD^+), cytoplasmic	Enzyme involved in glycolysis and lipid metabolism; no clear links to human disease although in vitro was identified as a potential pathway of interest in cancer therapeutics
Q8N142	ADSS1	Adenylosuccinate synthetase isozyme-1	Enzyme that catalyzes the first step in the conversion of IMP to AMP to regulate nucleotide levels; in rodents with cardiac hypertrophy, this gene is up-regulated; ADSS1 inactivation is also linked to tumor proliferation
Q9NZC2	TREM2	Triggering receptor expressed on myeloid cells-2	A receptor expressed on myeloid cells that is involves in immune function such as activating macrophages and inflammation; reduced activity of this protein is linked to Alzheimer's and dementia and further brain cell damage following ischemic stroke; increased expression of TREM2 is associated with Parkinson's disease.
Q16322	KCNA10	Potassium voltage-gated channel subfamily A member-10	A potassium gated channel that potentially mediates proximal tubule sodium reabsorption and vascular tone in the kidneys and the cardiac action potential; SNPs linked to increased susceptibility to CKD; mutation potentially responsible for inherited fatal cardiac arrhythmia
Q86SI9	C5orf38	Putative uncharacterized protein IRX2-DT	Identified as a molecular biomarker for coronary artery disease
P43307	SSR1	Translocon-associated protein subunit alpha	A transmembrane protein in the endoplasmic reticulum responsible for protein transport across the membrane; it is also involved in the biosynthesis of insulin (and potentially the pathogenesis of diabetes)
Q13508	ART3	Ecto-ADP-ribosyltransferase-3	ART3 is involved DNA repair, cell differentiation, and mediates the cell cycle; ART3 expression is associated with certain cancers such as melanoma and triple-negative breast cancer
A6NH11	GLTPD2	Glycolipid transfer protein domain-containing protein-2	A lipid transport protein; associated with increased CVD risk
Q03167	TGFBR3	Transforming growth factor beta receptor type 3	Involved in TGF-β signaling pathways that regulate cellular processes such as proliferation, migration, and homeostasis; TGFBR3 has been shown to be a tumor suppressor; in mouse model cardiac fibroblasts, it is also protective against apoptosis following hypoxic injury

AMP = adenosine monophosphate; CKD = chronic kidney disease; CVD = cardiovascular disease; IMP = inosine monophosphate; SNP = single nucleotide polymorphism.

patients with diabetes and coronary artery disease.[51] VE-cadherin was also found to be expressed in atherosclerotic lesions by endothelial cells and was associated with neovascularization.[52] Whether the association between VE-cadherin and PP is mediated through aortic atherosclerosis or whether it involves a medial aortic layer effect independent of atherosclerosis remains to be determined.

We also identified TAGLN, an actin-crosslinking protein that was shown to be abundantly expressed in smooth muscle cells and is an early marker of smooth cell differentiation. TAGLN was previously identified as a regulator of angiogenesis, and its expression was increased in elongating VE cells in animal model studies.[53] TAGLN has been previously shown to be expressed in abdominal aortic aneurysms and in calcific aortic valve disease,[54] but ours is the first study to highlight a link between TAGLN and LAS in humans. TAGLN has been shown to regulate arterial wall inflammation and chondrogenic conversion of vascular smooth muscle cells in mouse models.[55-57] TAGLN may therefore affect aortic wall stiffness through effects on vascular smooth muscle cell tone, aortic matrix remodeling and calcification.

Notch1 serves as an endothelial mechanosensor that is highly responsive to shear stress, facilitating divergent Notch activation across specific regions of the vascular wall and concurrently reducing inflammation.[58] Studies in mice demonstrated that the absence of Notch1 in smooth muscle cells restricts abdominal aortic dilation by preserving a contractile smooth muscle cell phenotype and impeding matrix remodeling, thereby thwarting the progression of abdominal aortic aneurysm.[59] Therefore, Notch1 may also in regulate LAS in the absence of aortic aneurysms via its effects on vascular smooth muscle cells and medial layer matrix remodeling. Our findings motivate further investigations of the mechanistic

pathways linking NOTCH1 with LAS and its potential as a therapeutic target.

HTRA1, a serine protease, has a diverse range of targets, including extracellular matrix proteins. It acts as a key regulator of matrix mineralization in vascular smooth muscle cells, via inhibition of TGF-β/BMP signaling and cleavage of specific matrix proteins.[60] HTRA1 is also essential for maintaining vascular maturation and homeostasis by regulating Notch and TGF-β signaling pathways.[61] HTRA1 degrades extracellular matrix components, playing a role in matrix mineralization, impairs elastogenesis, and contributes to the fragmentation of elastic fibers.[62,63] HTRA1 has also been shown to be involved in cerebral autosomal-recessive arteriopathy with subcortical infarcts and leukoencephalopathy,[64,65] a disorder characterized by small-vessel disease in the brain,[66] and in age-related macular degeneration.[67-69] This association highlights the importance of understanding the diverse roles of HTRA1 in both physiological and pathologic processes. Interestingly, HTRA1 antagonists are now available and being tested for the treatment of patients with age-related macular degeneration. Whether HTRA1 represents a therapeutic target for preventing or reducing LAS remains to be determined.

In addition to these known regulators of arterial structure, our study identified CILP2 to be associated with LAS. CILP2 has been previously associated with insulin resistance and coronary artery disease, but the underlying mechanistic basis is not well understood.[70,71] In addition to CILP2, our study identified several novel proteins (RARRES2, MANSC1, INHBC, SELL, CGA, GHR, and NEGR1) without known roles in arterial wall remodeling (Supplemental Table 6). Our findings should therefore be followed by dedicated mechanistic studies to understand the biologic role of these proteins in the vascular wall.

STUDY STRENGTHS AND LIMITATIONS. To our knowledge, this is the first study to investigate proteomic correlates of LAS and putative causal effects for 7,288 proteins leveraging robust instrumental variable analyses to overcome biases arising from confounding and reverse causation. The combination of discovery analyses in a large population (Asklepios cohort) with CF-PWV measurements (considered the reference metric of LAS) and the application of genetic epidemiologic methods increase the robustness and generalizability of our findings and are important strengths of our study. We applied methods to increase robustness against horizontal pleiotropy, restricted MR analyses to *cis*-acting genetic instruments, and conducted colocalization analyses to ensure that MR assumptions were not violated through confounding by linkage disequilibrium. These methods increase the confidence in inferences drawn from MR, which support potential causal effects of genetically predicted protein levels on LAS. Importantly, we relied on CF-PWV (the reference method to assess LAS) to identify candidate proteins in the Asklepios study, which enrolled a middle-aged population. This is important because previous studies have shown that in this age range, large artery stiffening precedes frank increases in PP. Indeed, in the Asklepios study, it was shown that the age-related increase in CF-PWV was not paralleled by an increase in arterial pulsatile impedance.[26] Similarly, Campos-Arias et al[72] more recently showed that in this age range, increases in CF-PWV over time are not paralleled reductions arterial volume compliance or aortic characteristic impedance (both of which are key determinants of PP). Indeed, surprisingly, aortic characteristic impedance even decreased slightly over time in younger subjects. There is therefore an age-dependent modulation of the effect of arterial wall stiffness on PP, whereby increases in PWV occur in middle age, whereas frank increases in PP occur later in life[27,72] as aortic wall stiffening overcomes compensatory pulsatile hemodynamic effects of outward aortic remodeling on characteristic impedance, volume compliance, and consequently on PP. Of note, UK Biobank participants are older (49-75 years at enrollment), and in this age range, PP is a highly clinically relevant phenotype occurring as a result of aortic wall stiffening. Our study was also subjected to a number of limitations, and findings should be interpreted with caution. First, our discovery was based on a middle-aged population of predominantly European ancestry and may not be generalizable to other ethnic groups. This population demonstrated a relatively low prevalence of diabetes, and its body weight distribution may not be representative of other populations more significantly affected by the epidemic of obesity. In addition, some proteins that may have causal roles on LAS may not have been identified because of power limitations in the Asklepios study (given our strict correction for multiple comparisons) or because of measurement errors, introducing noise in the measurements. In addition, plasma measurements of protein levels may not adequately represent biologic processes in the arterial wall. For instance, COL1A1 was negatively association with CF-PWV, but whether this reflects deposition and/or degradation of mature collagen 1 in tissue remains unclear. Moreover, considering that the SomaScan platform uses a single binder and direct readout, specificity may be compromised for each of the 7,288 proteins. Future work should focus on

verifying these findings with alternative technologies (antibody- or mass spectrometry–based methods) as a validation step. These results should be examined with caution and should be considered in conjunction with existing evidence of protein associations in previous studies and as a discovery in the case of novel protein associations. Additionally, we used a commercially available pathway analysis package, the basis of which is not publicly available for scrutiny. MR analyses rely on a number of assumptions that affect the reliability of causal inference. It is important to note that our findings do not provide evidence that targeting these proteins with therapeutic interventions will prove to be clinically beneficial. To further characterize the causal mechanisms and effects of protein inhibition or stimulation on LAS, further investigations are needed, including thorough analyses of drug targets and validation in model systems, followed by clinical studies as appropriate. Our analyses used CF-PWV to identify proteins associated with LAS. However, given that lack of availability of robust genetic instruments for CF-PWV, we used PP, a less adequate proxy of LAS, for our MR analyses. We did not assess the potential modifying effect of sex and other factors on the relationship between the plasma proteome and LAS, which should be the focus of future research. Finally, we note that relationships between plasma proteins found in our study and CF-PWV are relatively weak. However, we note that this may not necessarily correspond to the biologic importance of a given protein or pathway. First, plasma protein levels may be weak and/or variable proxies for corresponding biologic processes in tissue. Second, measurement error and variability, in both plasma protein levels and CF-PWV will weaken associations. Third, CF-PWV is a composite of long-term changes, whereas plasma protein levels were taken at a single time in a snapshot fashion, which could weaken observed associations even if underlying biologic links are important. Finally, these weak relationships may result from the complexity of the pathophysiologic determinants of LAS. Further work will be needed to assess the clinical impact of manipulating these pathways on the progression of LAS.

CONCLUSIONS

In this study, we identified 106 novel proteins that may serve as biomarkers of LAS, playing roles in tissue remodeling, inflammation and metabolic dysfunction. Using complementary genetic epidemiological methods, our causal inference analyses identified 13 candidate proteins with a potential causal association with pulse pressure, which should be prioritized for further investigation. The identification of these novel proteins underscores the need for proteomic screening to identify and prioritize potential therapeutic targets and accelerate the development of interventions to reduce LAS and downstream target organ damage.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Our study identified 106 novel proteins that may serve as biomarkers of LAS, playing roles in aortic wall remodeling and inflammation and metabolic dysfunction. Our causal inference analyses identified 13 potential candidates for further investigation. Our findings also shed light on the importance of targeting arterial stiffness as a discrete endpoint to prevent cardiovascular events, going beyond traditional risk factors such as hypertension and dyslipidemia, which do not explain the full pathophysiology of LAS.

TRANSLATIONAL OUTLOOK: The discovery of novel LAS biomarkers highlights the importance of performing proteomic screening to identify novel therapeutic targets and facilitate the early diagnosis of LAS and downstream target organ damage.

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APPENDIX For supplemental tables, please see the online version of this paper.