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ORIGINAL ARTICLE
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Circulating proteins and peripheral artery disease risk: observational and Mendelian randomization analyses

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Aims We conducted observational and Mendelian randomization (MR) analyses to explore the associations between blood proteins and risk of peripheral artery disease (PAD).

Methods and results The observational cohort analyses included data on 257 proteins estimated in fasting blood samples from 12 136 Swedish adults aged 55–94 years who were followed up for incident PAD via the Swedish Patient Register. Mendelian randomization analyses were undertaken using *cis*-genetic variants strongly associated with the proteins as instrumental variables and genetic association summary statistic data for PAD from the FinnGen study (11 924 cases and 288 638 controls) and the Million Veteran Program (31 307 cases and 211 753 controls). The observational analysis, including 86 individuals diagnosed with incident PAD during a median follow-up of 6.6-year, identified 13 proteins [trefoil factor two, matrix metalloproteinase-12 (MMP-12), growth differentiation factor 15, V-set and immunoglobulin domain-containing protein two, N-terminal prohormone brain natriuretic peptide, renin, natriuretic peptides B, phosphoprotein associated with glycosphingolipid-enriched microdomains one, C-C motif chemokine 15, P-selectin, urokinase plasminogen activator surface receptor, angiopoietin-2, and C-type lectin domain family five member A] associated with the risk of PAD after multiple testing correction. Mendelian randomization analysis found associations of T-cell surface glycoprotein CD4, MMP-12, secretoglobin family 3A member 2, and ADM with PAD risk. The observational and MR associations for T-cell surface glycoprotein CD4 and MMP-12 were in opposite directions.

Conclusion This study identified many circulating proteins in relation to the development of incident PAD. Future studies are needed to verify our findings and assess the predictive and therapeutic values of these proteins in PAD.

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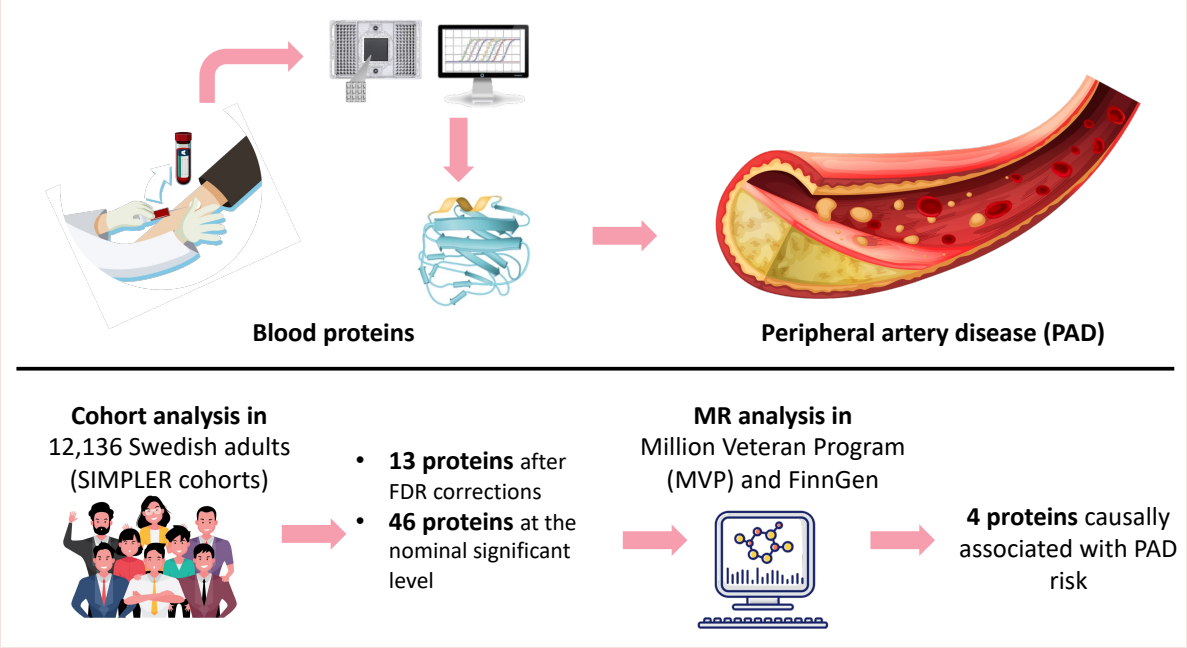
Graphical Abstract



Keywords Cohort • Mendelian randomization • Protein • Peripheral artery disease

Introduction

Peripheral artery disease (PAD) is a common vascular disease with a high incidence[1] and causes a large disease burden, especially in high-income countries.[2] Several modifiable factors, such as smoking, diabetes, hypertension, and concomitant cardiovascular diseases (CVDs), have been associated with the development of PAD.[2,3] There are some treatments for this disease, like statin therapy, P2Y12 inhibitors, low-dose rivaroxaban, vorapaxar, and cilostazol, supervised exercise therapy, and revascularization for PAD patients with severe pain. Proteins principally regulating molecular pathways have been highlighted for drug development. With multiplex methods, circulating proteins can be efficiently measured in a large sample with high accuracy. Several studies have investigated the associations of blood proteins with risk of coronary atherosclerosis.[4–7] However, there is a scarcity of data on the associations between blood proteins and the risk PAD.

Employing genetic variants as an instrument for the exposure, Mendelian randomization (MR) analysis can strengthen the causal inference by minimizing confounding and reverse causation.[8] This approach resembles the design of randomized controlled trials. In detail, given that genetic variants are randomly assorted at conception, MR based on genetic variants as the instrumental variable for the exposure can assign the participants into groups by natural randomization. The exposure proxied by genetic instruments is not associated with confounders. This process mimics randomization of randomized controlled trials and thus minimizes confounding. In addition, since the germline genotype is fixed and cannot be modified by onset or progression of disease, MR design can also diminish the influence of reverse causation. This method has been widely employed to assess the causality of associations and has been found to be valid as randomized controlled trials.[9–11] For MR investigations on a protein, one or more *cis*-genetic variants (i.e. a genetic variant within the gene region that encodes the protein) is usually utilized as the instrumental variable. Compared to using *trans*-genetic variants, using *cis*-variants as an instrumental variable is more likely to reflect protein-specific effects and thus satisfy the three key assumptions of MR that are (i) the selected genetic variant strongly associated with the protein, (ii) the used genetic variant for the protein are not associated with confounders, and (iii) the genetic variant affects the outcome only via the exposure.[12] Here, we conducted cohort and MR analyses to explore the associations of cardiovascular and cardiometabolic proteins with risk of PAD.

Methods

Study design

We explored the observational associations between 257 blood proteins and the risk of incident PAD in two Swedish cohorts. To strengthen the causality of the protein-PAD associations, MR analyses were conducted using data from large-scaled genetic studies.

Observational analysis

Study population

The observational analysis was based on data from two clinical sub-cohorts of the Swedish Infrastructure for Medical Population-Based Life-Course and Environmental Research (SIMPLER) that includes the Swedish Mammography Cohort (SMC) and Cohort of Swedish Men (COSM). Fasting blood samples were collected in clinical examinations by trained nurses during 2003–2009 and 2010–2019 for sub-cohorts of SMC and COSM, respectively. Meanwhile, participants were asked to fill in some questionnaires on diet, health status, and lifestyle. In total, 12 314

participants were recruited in two sub-cohorts. After removing 178 people with baseline PAD diagnosed before the day of blood sample collection, we included 12 136 participants in the analysis. Detailed information on cohorts and used questionnaires can be found on the SIMPLER website (https://www.simpler4health.se/).

Proteomic profiling

Venous blood samples were collected after an overnight fast and immediately centrifuged and stored at −80°C until analysis. In total, 276 plasma protein biomarkers were analysed using three high-throughput multiplex immunoassays: the Olink Proseek Multiplex CVD II, CVD III, and Metabolism (Olink Bioscience, Uppsala, Sweden), where the levels of protein expression were normalized on a log2 scale standardized per analysis plate. Values below limit of detection (LOD) were provided by the manufacturer and used as a protein selection criterium. The analyses were performed at SciLifeLab, Uppsala University, Sweden.[13] In this analysis, 19 proteins with more than 50% samples below the LOD were excluded (see Supplementary material online, *Table S1*). A small portion of specimens was set to missing given the analysed sample did not pass the manufacturer's quality control (3.6%, 0.8%, and 0.7% for CVD II, CVD III, and Metabolism panels, respectively). Included 257 proteins can be found in the Supplementary material online, *Table S2*.

Case ascertainment and follow-up

Incident PAD cases were ascertained by a medical diagnosis of PAD as the primary or contributing causes with diagnostic data obtained from the Swedish National Patient Register. The diagnostic codes used are shown in Supplementary material online, *Table S3*. The Swedish National Patient Register covers nearly all information on hospital-based inpatient and outpatient care.[14] We obtained date of death from the Swedish Death Registry. Participants were followed up from the baseline until the date of diagnosis of PAD, date of death, or end of follow-up (i.e. 31 December 2019), whichever came first.

Measurements of covariates

We obtained data on age, sex, education attainment, smoking, alcohol consumption, physical activity, and dietary intake from self-reported questionnaires. Diet quality was assessed by a modified Dietary Approaches to Stop Hypertension (mDASH) score.[15] Weight, height, estimated glomerular filtration rate (eGFR), levels of blood lipids and glucose, and blood pressure were measured by trained nurses in the health exam. Body mass index (BMI) was calculated by weight (in kg) divided by square of height (in m). Baseline diagnosis of cardiovascular disease (CVD) including coronary artery disease, heart failure, stroke, and atrial fibrillation was extracted from the Swedish National Patient Register. Detailed information of covariates is shown in Supplementary material online, *Table S4*.

Cox regression analyses

We used multiple imputation by chained equations (20 imputation cycles) to impute missing values of protein and covariates. The associations between circulating proteins and the risk of incident PAD were calculated by Cox proportional hazards regression model with age as the underlying time scale. The assumption of proportionality was examined by Schoenfeld residuals and found to be met. Two models were used: Model 1 adjusted for sex and plate, and Model 2 adjusted for sex, plate, BMI,[16] educational attainment,[17] baseline CVD,[18] smoking status,[19] alcohol consumption,[3] physical activity,[3] mDASH score,[3] eGFR,[20] low- and high-density lipoprotein cholesterol (LDLC and HDLC),[21] triglycerides,[21] systolic blood pressure,[21] and blood glucose levels.[22] Traits included in the Model 2 have been associated with PAD; however, it remains unclear whether all these are associated with blood proteins. In a conservative way of minimizing confounding, we adjusted for these factors in the Model 2. To examine the robustness of the associations, we conducted a sensitivity analysis among individuals with ($n = 2617$) and without ($n = 9519$) baseline CVD separately. The statistical tests were two-sided, and the analyses performed in Stata/SE (version 15.0; StataCorp, Texas, USA). The false discovery rate (FDR) based on Benjamini–Hochberg method was used to account for multiple testing. False discovery rate < 0.05 was considered statistically significant.

Mendelian randomization analysis

Peripheral artery disease data sources

Summary-level genetic data on PAD were obtained from the FinnGen R7 data release that included 11 924 participants with and 288 638 participants without PAD[23] and from a genome-wide association analysis in the Million Veteran Program (MVP) comprised of 31 307 participants with and 211 753 participants without PAD.[24] Detailed information (e.g. case definition and covariate adjustment) of the data sources is presented in Supplementary material online, *Table S5*.

Genetic instrument selection

To examine the causality of the association between proteins and PAD and reduce the possibility of Type 2 error caused by a heavy multiple-testing burden in cohort analysis, we included proteins associated with PAD at the nominal significance level in the cohort analysis to MR analysis. We used *cis*-genetic variants associated with proteins at the genome-wide significance threshold ($P < 5 \times 10^{-8}$) as instrumental variables for circulating proteins. The genetic associations (β coefficients and standard errors) with proteins were obtained from six genome-wide association studies, where protein profiles were analysed by SomaLogic assay in four studies[25–28] and Olink in two studies.[29,30] Lead *cis*-genetic variants provided by five studies[25–28,30] and single nucleotide polymorphisms (SNPs) in the protein-coding gene region with the pruning threshold of $R^2 < 0.01$ from Folkersen *et al.* study[29] were used. Single nucleotide polymorphisms for the identical protein from different studies were treated as separate instruments and independently used in MR analysis with the aim of mutual verification. Missing SNP was replaced by a proxy SNP with strong linkage disequilibrium ($r^2 \geq 0.8$), and proteins without genetic instruments were removed from the analysis. Detailed information on used studies is presented in Supplementary material online, *Table S6*. Instrument variables are listed in Supplementary material online, *Table S7*.

Reverse Mendelian randomization analysis for matrix metalloproteinase-12

In this analysis, genetic liability to PAD was deemed the exposure and levels of matrix metalloproteinase-12 (MMP-12) the outcome. We selected genetic variants associated with PAD at the genome-wide significance level in the MVP comprised of 31 307 participants with and 211 753 participants without PAD.[24] After pruning genetic variants with linkage disequilibrium $r^2 < 0.01$, we selected 18 SNPs as an instrumental variable for PAD. Summary-level data on MMP-12 were extracted from the SCALLOP consortium (Folkersen *et al.* study[29]) where blood proteins were measured using the Olink platform in > 30 000 participants.

Mendelian randomization statistical analysis

We calculated F statistic to assess the strength of the genetic instrument. The F statistic >10 indicates a good strength of minimal weak instrument bias.[31] Mendelian randomization associations were estimated by the Wald ratio method (i.e. ratio estimate equals the β coefficient for the effect of the SNP on PAD divided by the β coefficient for the effect of the SNP on the protein).[32] The standard error of the ratio estimate was estimated using the delta method.[32] For proteins with two or more SNPs from Folkersen *et al.* study,[29] the inverse variance weighted method was used to estimate MR associations with PAD. Mendelian randomization estimates for each protein from the FinnGen and the MVP studies were combined using the fixed-effect meta-analysis method. We performed two sensitivity analyses, the weighted median and MR–Egger methods, for proteins with greater than or equal to three *cis*-SNPs as instrumental variables to examine the consistency of the results and detect potential horizontal pleiotropy. The weighted median method assumes that more than half of the weight is from valid SNPs and thus generate consistent MR estimates. The MR–Egger regression method can detect horizontal pleiotropy by its intercept test and provide estimates after correction for potential horizontal pleiotropy; however, the method is usually underpowered. The analyses were conducted using TwoSampleMR and MendelianRandomization packages in R software 4.1.1.

Results

Observational analysis of proteins and peripheral artery disease

During a median follow-up of 6.4 (the interquartile range of 8.5) years, 86 participants developed incident PAD diagnosed. Baseline characteristics of participants by incident PAD status are shown in *Table 1*. In brief, compared to individuals without incident PAD diagnosis, those with incident PAD were more likely to be men and had more baseline traditional CVD risk factors.

In total, plasma levels of 13 out of 257 proteins were associated with the risk of incident PAD after FDR correction in Model 1 adjusted for age, sex, and plate (*Figure 1* and *Figure 2*). Per standard deviation (SD) increase in circulating protein levels, the hazard ratio (HR) of PAD was 1.65 [95% confidence interval (CI) 1.39–1.97] for TFF2 (trefoil factor 2), 1.63 (95% CI 1.34–1.99) for MMP-12, 1.56 (95% CI 1.27–1.91) for GDF-15 (growth differentiation factor-15), 1.49 (95% CI 1.21–1.82) for VSIG2 (v-set and immunoglobulin domain-containing protein 2), 1.49 (95% CI 1.21–1.83) for NT-proBNP (N-terminal prohormone brain natriuretic peptide), 1.49 (95% CI 1.21–1.85) for REN (renin), 1.42 (95% CI 1.17–1.72) for BNP (natriuretic peptides B), 1.39 (95% CI 1.16–1.66) for PAG1 (phosphoprotein associated with glycosphingolipid-enriched microdomains 1), 1.39 (95% CI 1.15–1.67) for CCL15 (C-C motif chemokine 15), 1.38 (95% CI 1.13–1.68) for SELP (P-selectin), 1.38 (95% CI 1.12–1.7) for CLEC5A (C-type lectin domain family 5 member A), 1.36 (95% CI 1.13–1.64) for ANGPT2 (angiopoietin-2), and 1.32 (95% CI 1.11–1.57) for U-PAR (urokinase plasminogen activator surface receptor). After further adjustment for lifestyle and clinical factors in Model 2, the associations for all proteins except for U-PAR remained significant (*Figure 2*). These associations remained overall consistent in the sensitivity analysis in individual with and without baseline CVD albeit with larger CIs (see Supplementary material online, *Table S8*). There were 33 associations with the nominal $P < 0.05$ and FDR-adjusted $P > 0.05$ (see Supplementary material online, *Table S9*).

Mendelian randomization analysis of the protein–peripheral artery disease association

We conducted MR analyses for 46 proteins associated with PAD at the nominal significant level in the Cox regression analysis adjusted for age, sex, and plate. Seven proteins were removed from the analysis due to without suitable instrumental variables, and one protein (LEG9, galectin-9) was excluded from the analysis in FinnGen due to missing without a proxy SNP. All *F* statistics for used genetic instruments were >10. Genetically predicted levels of MMP-12, CD4 (T-cell surface glycoprotein CD4), and GDF-15 were associated with PAD risk in FinnGen (*Figure 3*). For genetically predicted per SD increase, the odds ratio (OR) of PAD was 0.93 (95% CI 0.89–0.97) for MMP-12, 1.13 (95% CI 1.00–1.29) for GDF-15, and 0.89 (95% CI 0.82–0.96) for CD4. In the MVP study, there were additional associations for IL-1ra (interleukin-1 receptor antagonist protein), ADM, CTSD

Table 1 Baseline characteristics of 12 136 Swedish adults by incident peripheral artery disease (PAD) status during follow up

Characteristic	Without incident PAD (*n* = 12 050)	Incident PAD (*n* = 86)	*P* for difference
Age in year	71.5 ± 6.8	74.5 ± 6.7	<0.001
Male, *n* (%)	4593 (38.1)	44 (51.2)	0.013
Body mass index in kg/m^2	26.4 ± 4.2	25.9 ± 3.1	0.083
Education attainment ≥12 years, *n* (%)	3443 (28.6)	17 (20.0)	0.072
Baseline cardiovascular disease, *n* (%)	2583 (21.4)	34 (39.5)	<0.001
Coronary artery disease, *n* (%)	70 (0.6)	16 (1.6)	<0.001
Heart failure, *n* (%)	74 (0.7)	12 (1.5)	0.008
Stroke, *n* (%)	84 (0.7)	2 (0.5)	0.608
Atrial fibrillation, *n* (%)	70 (0.7)	16 (1.2)	0.026
Current smoker, *n* (%)	4181 (34.7)	54 (62.8)	<0.001
Excessive alcohol consumption, *n* (%)	1406 (11.8)	11 (12.8)	0.773
Physical activity, *n* (%)			0.023
<10 min/day	685 (5.7)	5 (5.8)	
10–30 min/day	1391(11.5)	19 (22.1)	
30–60 min/day	5385 (44.7)	35 (40.7)	
>60 min/day	4589 (38.1)	27 (31.4)	
mDASH score	18.1 ± 3.7	17.8 ± 3.5	0.327
eGFR in mL/min/1.73m^2	81.0 ± 15.2	75.6 ± 17.9	<0.001
LDLC in mmol/L	3.3 ± 1.0	3.4 ± 1.0	0.303
HDLC in mmol/L	1.5 ± 0.4	1.4 ± 0.3	0.025
Triglyceride in mmol/L	1.3 ± 0.7	1.5 ± 0.7	0.021
Glucose in mmol/L	5.7 ± 1.4	6.1 ± 1.6	0.011
Systolic blood pressure in mmHg	138.9 ± 17.6	146.1 ± 20.3	<0.001

eGFR, estimated glomerular filtration rate; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; mDASH, modified Dietary Approaches to Stop Hypertension; PAD, peripheral artery disease; continuous and categorical variables were expressed in mean (standard deviation) and *n* (%), respectively.

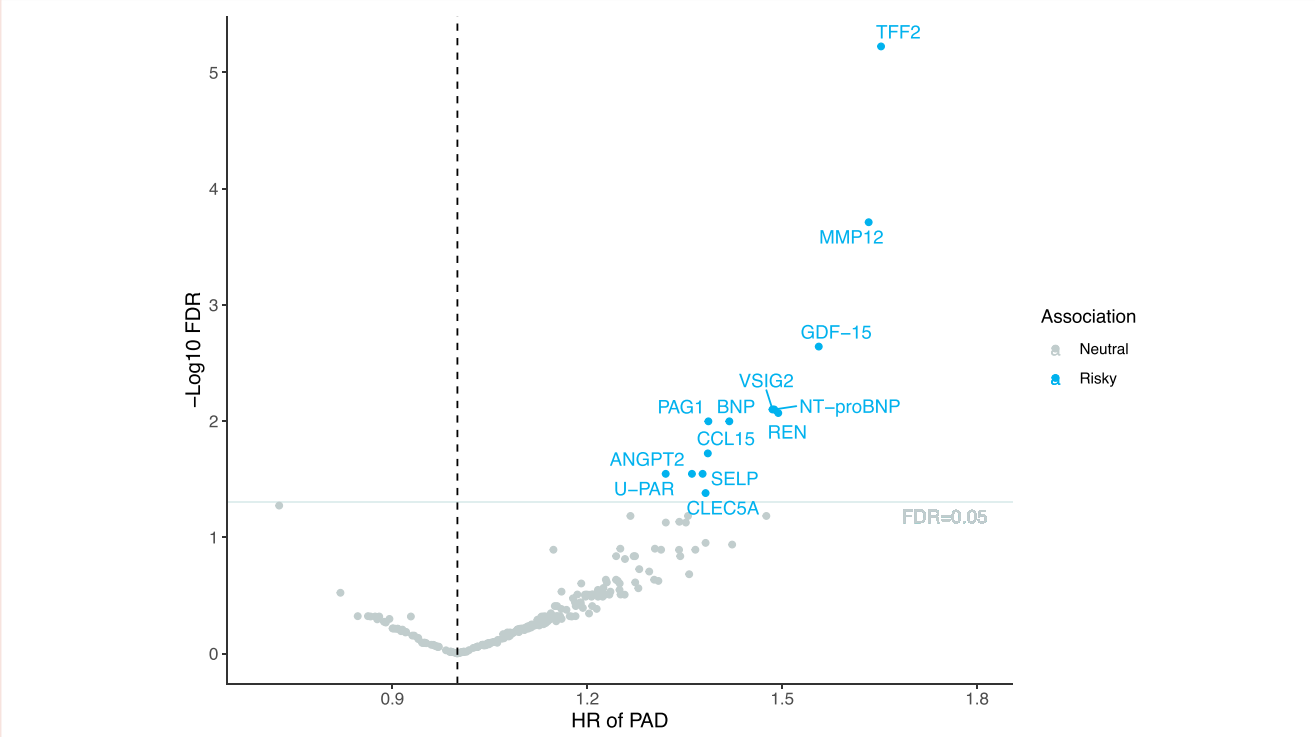


Figure 1 Volcano plot of the observational associations between 257 proteins and the risk of incident peripheral artery disease (PAD) in the Swedish cohort analysis. FDR, false discovery rate; HR, hazard ratio. The associations were derived from the model adjusted for age, sex, and plate. Full name of proteins can be found in Supplementary material online, *Table S2*.

(cathepsin D), and SCGB3A2 (secretoglobin family 3A member 2) (*Figure 3*). In the combined analysis of MR estimate in FinnGen and MVP studies, genetically predicted levels of CD4 (OR 0.90; 95% CI 0.83–0.96), MMP-12 (OR 0.91; 95% 0.88–0.93), SCGB3A2 (OR 1.08; 95% CI 1.00–1.17), and ADM (OR 1.16; 95% CI 1.05–1.27) were associated with the risk of PAD (*Figure 3*). These associations were generally consistent using genetic instrumental variables from different studies (see Supplementary material online, *Table S10*). For proteins with SNPs greater than or equal to three, the associations were consistent in the sensitivity analysis, and we detected no indication of horizontal pleiotropy in MR–Egger intercept test ($P > 0.05$; Supplementary material online, *Table S11*). There were no associations of genetically predicted levels of other studied proteins with PAD risk (see Supplementary material online, *Table S10*). Of note, the observational and MR associations for MMP-12 and CD4 were in the opposite direction (*Figure 4*).

Reverse Mendelian randomization analysis for matrix metalloproteinase-12

Genetic liability to PAD was not associated with levels of MMP-12 (see Supplementary material online, *Table S12*). The null association was observed in sensitivity analyses. There was heterogeneity among SNPs' estimates but no horizontal pleiotropy (*P* for MR–Egger intercept = 0.89).

Discussion

The present study identified 13 circulating proteins that were observationally associated with PAD incidence in Swedish adults. Mendelian randomization analysis in two populations of Finnish and US adults, including a total of 43 231 PAD cases and 500 391 controls, revealed associations of CD4, MMP-12, SCGB3A2, and ADM with PAD risk. However, the associations for CD4 and MMP-12 were in opposite directions in the observational and MR analyses. The reverse MR found limited data in support of an association between genetic liability to PAD and MMP-12 levels.

A study including data from six US community-based cohorts estimated the residual lifetime risk of PAD in relation to many traditional risk factors for CVD.[33] The direction of associations for identified proteins in this study were comparable to that of most risk factor for PAD, like diabetes, SBP, and hyperlipidaemia.[33] This comparison implies the vital roles of identified circulating proteins in the development of PAD, which warrants more explorations for PAD prevention and treatment using these protein targets.

High levels of MMP-12 have been associated with an increased risk of several atherosclerotic cardiovascular conditions, such as atherosclerotic media destruction and ectasia,[34] intima-media thickness in the common carotid artery (IMT-CCA) and in the bulb (IMT-bulb),[5] and incident cardiovascular events[4,35] in some but not all[36] observational studies. Our observational analysis for the first time revealed a positive association between MMP-12 and PAD in which atherosclerosis plays a vital role. The mechanisms in support of this positive association may be related to lesion formation, plaque stability, reduced coagulation, and perivascular fibrosis.[37,38] However, the MR analysis identified a strong albeit opposite effect of MMP-12 on PAD, which is in line with previous MR findings of inverse associations of MMP-12 with the risk of coronary artery disease[28] and ischaemic stroke.[4,39] Even though the direction of the association was discordant between observational and MR analyses, these findings pointed out a possible causal role of MMP-12 in the development of atherosclerotic outcomes. The reason of this discrepancy is unclear but possibly related to the feedback mechanism,[4] which

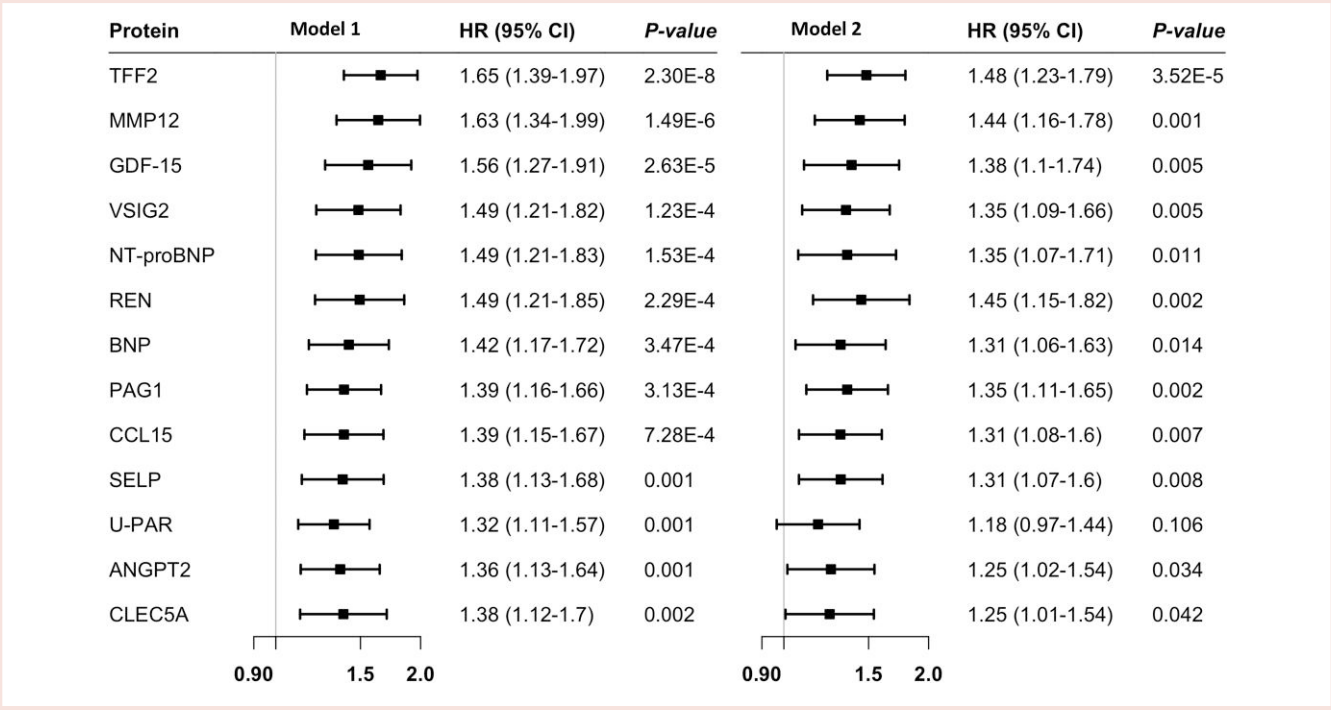


Figure 2 The observational associations between 13 proteins and the risk of incident peripheral artery disease with false discovery rate <0.05. CI, confidence interval; HR, hazard ratio. Model 1 adjusted for age, sex, and plate; Model 2 adjusted for age, sex, body mass index, plate, education attainment, baseline cardiovascular disease, smoking status, alcohol consumption, physical activity, mDASH score, eGFR, low- and high-density lipoprotein cholesterol, triglycerides, blood pressure, and blood glucose levels. Full name of proteins can be found in Supplementary material online, *Table S2*.

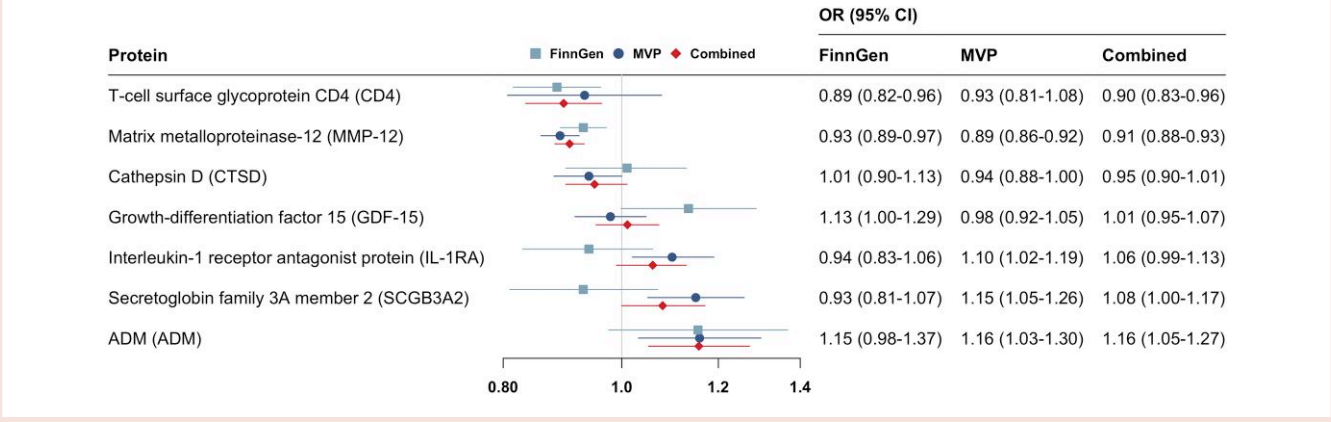


Figure 3 Potential causal associations of circulating proteins with risk of peripheral artery disease in Mendelian randomization analysis. CI, confidence interval; MVP, the Million Veteran Program; OR, odds ratio.

means that MMP-12 may be upregulated to compensate for proatherogenic processes during the early stage of atherogenesis[40] and thus individuals with reduced capacity to synthesize this protein have an increased cardiovascular risk. To test this hypothesis, we conducted an inverse MR analysis on the association of genetic liability to PAD with MMP-12 levels. However, the reverse MR analysis did not detect a significant association instead of an inverse trend in the primary MR analysis even though the analysis might be underpowered. Thus, if anything, this inverse link partly supports this hypothesis. Of note, confounding may also contribute to this disagreement. However, we measured and adjusted for many important risk factors in observational study although we could not completely rule out confounding caused by unmeasured factors.

Our observational and MR associations were also contradictive for the association between CD4 and PAD risk. This disagreement was also observed in previous studies. In some observational studies, a decline in CD4 count was associated with an elevated risk of endothelial dysfunction[41] and CVD.[42] However, lack of CD4 was shown to

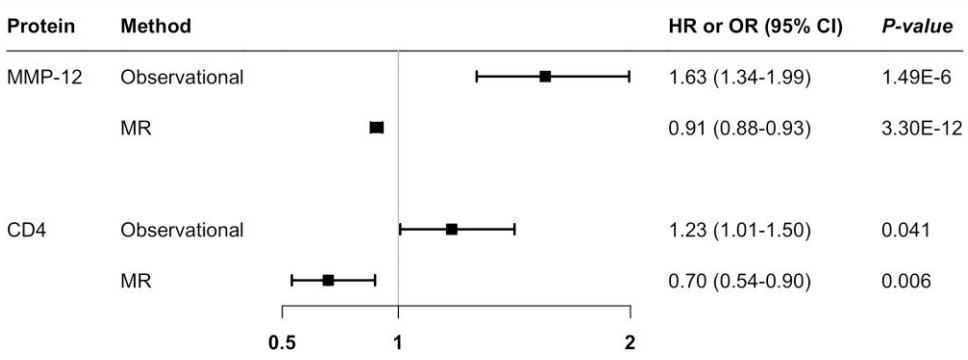


Figure 4 Contradictory associations of MMP-12 and CD4 with peripheral artery disease risk in observational and Mendelian randomization analyses. The observational associations were adjusted for age, sex, and plate. CI, confidence interval; CD 4, T-cell surface glycoprotein CD4; HR, hazard ratio; MMP-12, matrix metalloproteinase-12; OR, odds ratio; MR, Mendelian randomization.

substantially decreased the development of atherosclerosis in apolipoprotein E knockout mice.[43] As for the discrepancy in our study, the difference in the content actually measured in observational and MR analysis may be the reason. In observational study, serum CD4 protein was measured, and this protein in the circulation may be majorly derived from the membrane particles after CT4 T-cell death. However, CD 4 protein levels proxied by genetic variants may reflect more the amount of CD4+ T cells. Although there are no robust explanation for the disagreement, these findings suggest the complex roles of the adaptive immune response in the development of the atherosclerosis,[44] which may provide attractive targets for atherosclerotic prevention.

Evidence on the positive association between GDF-15 and atherosclerosis risk is consistent between studies.[45,46] Our study found a positive between GDF-15 and PAD in a generally healthy middle-aged population for the first time, and this association was partly strengthened in MR analysis in FinnGen instead of in the MVP study. Likewise, IL-1RA was positively associated with PAD risk in the cohort analysis and MR analysis in the MVP study, which is in line with previous findings.[47,48] Given that these associations were not consistently observed in our analyses using different sources, more studies are needed to verify these findings. In addition, the study found possible associations of CTSD and SCGB3A2 with PAD risk, which are novel findings that need confirmation. Our cohort analysis identified the association of previously established atherosclerosis-related proteins, such as NT-proBNP and U-PAR,[4] as well as novel proteins, such as VSIG2, REN, CCL15, and SELP, with incident PAD, but the MR analysis was not able to provide evidence for a causal relationship between these proteins and PAD. Nonetheless, these proteins may have utility as predictive biomarkers for PAD.

The strengths of our study include a large sample size with protein data, nearly complete follow-up information, inclusion of important covariates, and combination of traditional observational and MR analyses. At the same time, there are limitations that deserve to be emphasized when interpreting our results. First, the number of PAD cases in the observational analysis was limited despite the large overall cohort and a long follow-up. Thus, some weak associations might be missed due to inadequate power, and multiple correction might inflate the rate of Type 2 error. Second, our cohort findings among patients with baseline CVD might be affected by residual confounding from use of certain medications, like statins. However, we were able to adjust for baseline CVD diagnosis, which tightly correlated with corresponding medications. In addition, these associations remained overall consistent in individuals without baseline CVD who usually use few medications. Another confounding source is lipoprotein(a), an important risk factor for PAD,[49] which was not measured in the cohorts and thus could not be adjusted for in the analysis, even though whether lipoprotein(a), which is largely determined by variations in the *LPA* gene, is associated with studied blood proteins is unknown. Third, our observational and MR analyses were based on individual of European populations. Whether our findings can be generalized to other populations needs verification. Fourth, the proteomic platform used for the cohort study was targeted and incomplete in its coverage of potential proteins of interest, undoubtedly missing important potential protein biomarkers. Fifth, we could not replicate the results of the cohort analysis in an independent sample. However, the potential protein–PAD associations were tested by MR analyses in two populations with generally consistent findings although FinnGen population may have a different genetic background from the traditional European populations. Sixth, blood samples in the cohort analysis were collected at different timepoints and time in freezer might affect the levels of proteins, which might influence the results. However, an inflated variation in protein levels would attenuate the association towards to a null hypothesis in a conservative way. Seventh, horizontal pleiotropy could not be examined in MR analysis due to the lack of summary-level data even though this bias should be minimal when using *cis*-SNPs with a clear function as instrumental variables.

In summary, this study discovered many circulating proteins in relation to the development of PAD. These population-level findings may provide clues for future molecular exploration of mechanistical insights of PAD development. In addition, more studies are needed to assess the predictive and therapeutic values of these PAD-associated proteins.

Lead author biography

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Data availability

De-identified SIMPLER data are available for researchers upon application (http://www.simpler4health.se/). Summary-level data from the FinnGen study of PAD can be obtained via https://finngen.gitbook.io/documentation/. Summary data from the MVP GWAS of PAD can be obtained via dbGAP, accession code no. phs001672.v2.p1.

Supplementary material

Supplementary material is available at *European Heart Journal Open* online.

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Conflict of interest: SMD reported receiving grants from the US Department of Veterans Affairs during the conduct of the study and receiving grants from RenalytixAI and personal fees from Calico Labs outside the submitted work. All other authors have no conflicts to declare.

Author contributions

S.Y. and S.C.L. conceived and designed the study. S.Y. and O.E.T undertook the statistical analyses. S.Y. wrote the first draft of the manuscript. All authors provided important comments to the manuscript and approved the final version of the manuscript.

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