ESC European Society of Cardiology

European Journal of Preventive Cardiology (2023) **00**, 1–10
https://doi.org/10.1093/eurjpc/zwad087

FULL RESEARCH PAPER
Coronary artery disease

Prediction of coronary artery disease using urinary proteomics†

Dongmei Wei [1], Jesus D. Melgarejo [1], Lucas Van Aelst[2], Thomas Vanassche [2], Peter Verhamme [2], Stefan Janssens [2], Karlheinz Peter [3,4], and Zhen-Yu Zhang [1]*

[1]Studies Coordinating Centre, Research Unit Hypertension and Cardiovascular Epidemiology, KU Leuven Department of Cardiovascular Sciences, University of Leuven, Campus Sint Rafaël, Kapucijnenvoer 7, Box 7001, BE-3000 Leuven, Belgium; [2]Division of Cardiology, University Hospitals Leuven, University of Leuven, Herestraat 49, 3000 Leuven, Belgium; [3]Baker Heart and Diabetes Institute, 75 Commercial Rd, Melbourne VIC 3004, Australia; and [4]Department of Cardiology, The Alfred Hospital, 55 Commercial Rd, Melbourne VIC 3004, Australia

Received 12 January 2023; revised 13 March 2023; accepted 20 March 2023; online publish-ahead-of-print 21 March 2023

Aims Coronary artery disease (CAD) is multifactorial, caused by complex pathophysiology, and contributes to a high burden of mortality worldwide. Urinary proteomic analyses may help to identify predictive biomarkers and provide insights into the pathogenesis of CAD.

Methods and results Urinary proteome was analysed in 965 participants using capillary electrophoresis coupled with mass spectrometry. A proteomic classifier was developed in a discovery cohort with 36 individuals with CAD and 36 matched controls using the support vector machine. The classifier was tested in a validation cohort with 115 individuals who progressed to CAD and 778 controls and compared with two previously developed CAD-associated classifiers, CAD238 and ACSP75. The Framingham and SCORE2 risk scores were available in 737 participants. Bioinformatic analysis was performed based on the CAD-associated peptides. The novel proteomic classifier was comprised of 160 urinary peptides, mainly related to collagen turnover, lipid metabolism, and inflammation. In the validation cohort, the classifier provided an area under the receiver operating characteristic curve (AUC) of 0.82 [95% confidence interval (CI): 0.78–0.87] for the CAD prediction in 8 years, superior to CAD238 (AUC: 0.71, 95% CI: 0.66–0.77) and ACSP75 (AUC: 0.53 and 95% CI: 0.47–0.60). On top of CAD238 and ACSP75, the addition of the novel classifier improved the AUC to 0.84 (95% CI: 0.80–0.89). In a multivariable Cox model, a 1-SD increment in the novel classifier was associated with a higher risk of CAD (HR: 1.54, 95% CI: 1.26–1.89, $P < 0.0001$). The new classifier further improved the risk reclassification of CAD on top of the Framingham or SCORE2 risk scores (net reclassification index: 0.61, 95% CI: 0.25–0.95, $P = 0.001$; 0.64, 95% CI: 0.28–0.98, $P = 0.001$, correspondingly).

Conclusion A novel urinary proteomic classifier related to collagen metabolism, lipids, and inflammation showed potential for the risk prediction of CAD. Urinary proteome provides an alternative approach to personalized prevention.

Lay summary
- A biomarker that can predict coronary artery disease (CAD) is urgently in need.
- We developed and validated a urinary proteomic classifier for the prediction of CAD.
- The proteomic classifier involved in atherosclerosis improved the risk reclassification on top of the clinical risk score.

Keywords Coronary artery diseases • Proteomics • Urine • Collagen turnover • Atherosclerosis

Introduction

Coronary artery disease (CAD) is the most common heart disease affecting 126.5 million people and a leading cause of mortality, responsible for an estimated 8.9 million deaths worldwide in 2017.[1] Despite the advances in the management of modifiable risk factors, residual risk remains.[2] Given the considerable number of patients with CAD and the growing economic burden it causes,[1] the need for targeted intervention strategies is urgent. The development of targeted treatments requires insightful inputs into the mechanisms and biomarkers

* Corresponding author. Tel: +32-16-34-7104, Fax: +32-16-347106, Email: zhenyu.zhang@med.kuleuven.be

† All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

for CAD. Coronary artery disease can progress asymptomatically; thus, biomarkers that can detect the insidious ongoing pathophysiological process prior to clinical events and provide prognostic value are particularly in great demand.

Although blood is a common source of biomarker discovery for cardiovascular diseases,[3] urine, which can be collected noninvasively and easily, is another reservoir of biomarkers.[4] Proteins leaking or being secreted from multiple organs, including the cardiovascular system, can enter into the circulation and filter through the kidneys into the urine.[4,5] Therefore, a comprehensive urinary proteomics analysis can reflect systematic disease-associated changes, holds the promise to identify predictive biomarkers and uncover the mechanisms for cardiovascular diseases. We have previously reported urinary proteomic biomarkers for arterial stiffness,[6] vascular calcification,[7] and heart failure.[8,9] Previous multidimensional urinary proteomic biomarkers have also shown the potential for the detection and prediction of CAD.[10–13] For instance, a panel of 238 urinary peptides, CAD238, provided an area under the receiver operating characteristic curve (AUC) of 0.87 for the detection of CAD in 138 samples from 71 CAD patients and 67 healthy controls.[10,11] ACSP75 was developed for the prediction of acute coronary syndrome with an AUC of 0.66 in 42 cases and 42 controls.[13] Given the potential of urinary proteome analysis, there might be room for improvement of the classifier because the performance of a classifier may not be maintained when generalizing to a large cohort. Thus, this study aimed to develop a new urinary proteomic classifier for the prediction of CAD endpoints and validate its prognostic value in 893 individuals. Moreover, we hypothesized that urinary peptides discriminating CAD could provide insights into the pathological processes of CAD at an early stage; thus, we comprehensively described the pathways reflected by the identified urinary proteomic biomarkers with bioinformatic approaches.

Methods

Study design and participants

The study was a prospective study, comprised of a discovery cohort and a validation cohort (*Figure 1*). The discovery cohort consisted of 36 cases and 36 matched controls from a population-based study, the Flemish Study on Environment, Genes, and Health Outcomes (FLEMENGHO).[7] Cases were

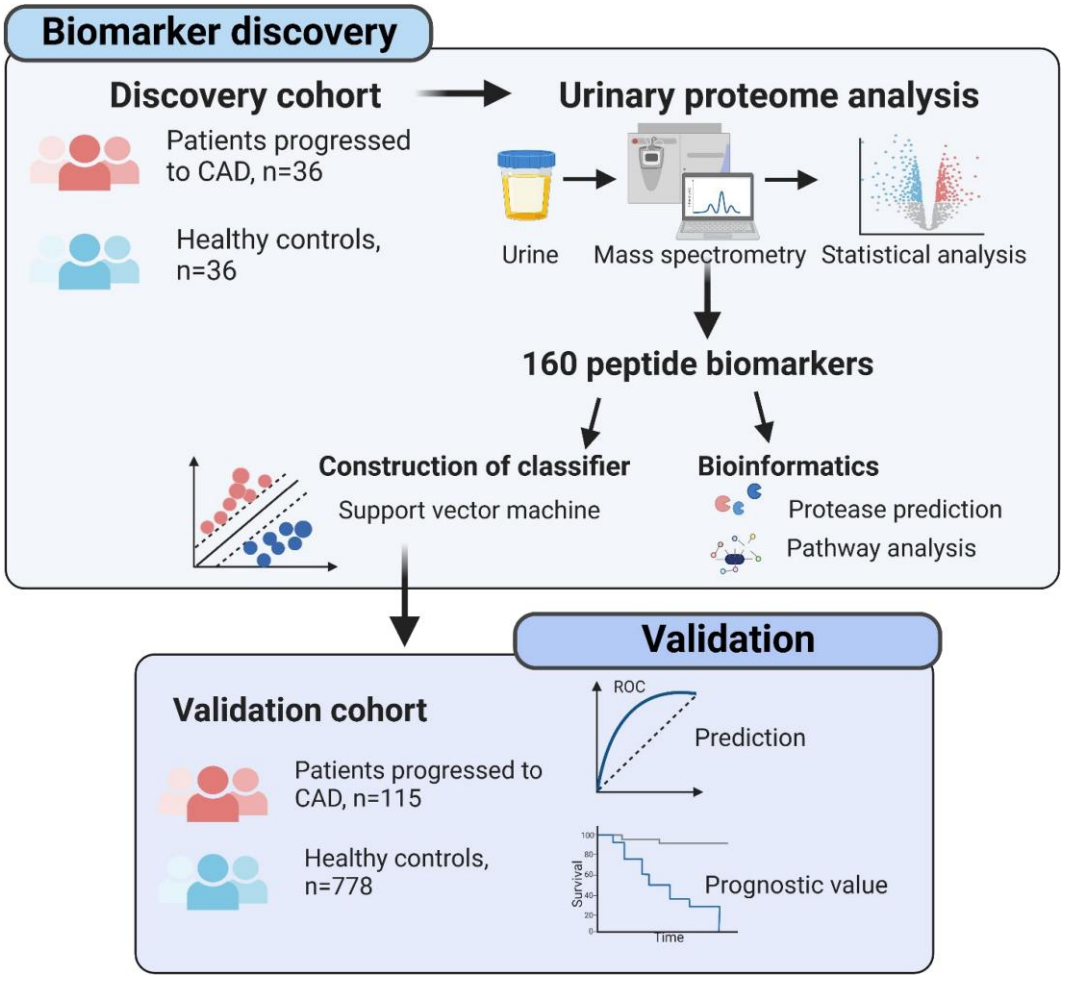


Figure 1 The schematic diagram of study design. In the biomarker discovery phase, urinary proteome analysis was performed on 36 patients who progressed to coronary artery disease and 36 matched controls. A total of 160 urinary peptides were identified to be significantly different between cases and controls, and their biological function was elucidated by bioinformatic analysis. Simultaneously, 160 peptides were used to construct a classifier for the discrimination of coronary artery disease by the supervised machine learning method, and the predictive performance and prognostic value were evaluated by an independent validation cohort. CAD, coronary artery disease.

individuals asymptomatic for CAD at baseline but progressed to CAD during a median of 8.3-year [interquartile (IQR): 5.5–9.8] follow-up. Coronary artery disease was defined as myocardial infarction, acute coronary syndrome, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, and any fatal ischaemic heart disease. Cases and controls with an averaged estimated glomerular filtration rate (eGFR) of 80.5 (IQR: 71.0–89.3) mL/min/1.73 m^2 were matched for sex, age, history of hypertension, antihypertensive treatment, and total cholesterol. The validation cohort was comprised of 893 individuals (115 with and 778 without CAD) from the FLEMENGHO study (35 with and 702 without CAD) or extracted from the Human Urinary Proteome Database (80 with and 76 without CAD), consisting of more than 85 000 samples from various clinical and research centres.[14]

The enrollment of the FLEMENGHO study (Belgium) started from 1985 to 2004, and the information on coronary events was collected until December 2016.[7] Participants extracted from the Human Urinary Proteome Database were part of four prospective studies with diverse clinical settings.[13,15–19] There were 34 participants (18 progressed to CAD and 16 did not) enroled in the Coronary Artery Calcification in Type I Diabetes (CACTI) study between 2000 and 2002 and followed up for over 2.4 years.[15,16] A total of 64 patients (32 progressed to CAD and 32 did not) with chronic kidney disease were from the outpatient clinic of the Nephrology section of the Ghent University Hospital (Belgium). They were recruited between 2011 and 2014 and followed up until June 2017.[17] Additionally, there were 28 participants (14 progressed to CAD and 14 did not) enroled in the Australian Diabetes, Obesity and Lifestyle Study (AusDiab) during 1999–2000 with a follow-up of 5 years.[13,18] Moreover, 30 patients with type 2 diabetes (16 progressed to CAD and 14 did not) were recruited from the outpatient clinic for diabetes and nephrology in Zwolle (The Netherlands) in 1998 and 2001 with a follow-up of 3.7 years.[19] All the latter data sets were fully anonymized and previously published, with respective references provided below. Demographic and clinical characteristics from extracted data sets included sex, age, hypertension, diabetes, office blood pressure, and eGFR. The second use of FLEMENGHO study data (B32220083510) was approved by the University of Leuven Ethics Committee and participants provided written informed consent. Data sets extracted from the Human Urinary Proteome Database were previously published, and relevant studies were conducted in compliance with the Helsinki declaration for research in humans and received an approval from the responsible review boards.[13,15–19] Coronary artery disease endpoint was defined as myocardial infarction, acute coronary syndrome, new-onset angina pectoris, ischaemic cardiomyopathy, and coronary revascularization. In the validation, 115 individuals experienced CAD endpoints, including 57 with new myocardial infarctions.

Urinary proteome analysis

Urinary proteome analysis was performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) coupled to a micrOTOF MS (Bruker Daltonics, Bremen, Germany). MosaFinder software was used to process mass spectral data and to generate a raw list of peptides or small proteins before being annotated according to prior sequenced peptides from the Human Urinary Proteome Database.[14] Peptide intensities were normalized using 29 collagen peptides, serving as an internal standards, being in general not affected by the disease, to assure comparability between different data sets.[20] Further information on sample preparation, data processing, and sequencing of the peptides was described elsewhere.[9,21,22]

Classifier construction

Distinct peptides between individuals who progressed to reach a CAD endpoint and controls were identified in the discovery cohort. Urinary sequenced peptides identified in ≥30% of either cases or controls were analysed. Peptide abundances were compared using the nonparametric Wilcoxon test, followed by adjustment for multiple testing. Peptides were considered statistically significant when the *P*-value was less than 0.05 after adjustment for multiple testing by Benjamini–Hochberg correction. The statistical validity of significant peptides was further confirmed by permutation analysis that randomly excluded 30% of the samples with 10 times repetition. Peptides with a nominal *P*-value of <0.05 in more than 50% of the permutation analyses were considered for further analysis. The support vector machine (SVM), a supervised machine learning algorithm, was applied to construct a classifier for the discrimination of CAD using the significant peptides. Model construction and parameter tuning were performed with the MosaCluster software.[23] The model's generalization was examined by take-one-out cross-validation.

Statistical analysis

Statistical analysis was conducted with SAS software, version 9.4 (SAS Institute, Cary, NC, USA). Means and percentages were compared using a *t*-test or analysis of variance (ANOVA) test or Fisher's test as appropriate. Statistical significance was considered as a two-sided *P*-value of 0.05. Time-dependent receiver operating characteristic (ROC) curves and the AUC were used to estimate the predictive capacity of the new classifier for incident CAD at 3, 5, and 8 years. The predictive capacity of previously developed classifiers, CAD238 and ACSP75, were assessed as comparisons.[10–13] The AUC estimate and 95% confidence interval (CI) for each classifier were calculated using the PHREG procedure to fit the Cox regression model in SAS. Moreover, whether the addition of the urinary proteomic classifier could improve risk reclassification for CAD on top of the Framingham or SCORE2 risk score was further evaluated. The Framingham risk score was calculated based on common clinical risk factors, including sex, age, systolic blood pressure, smoking, diabetes, treatment of hypertension, total cholesterol, and high-density lipoprotein cholesterol.[24] The improvement in risk reclassification was evaluated by net reclassification index (NRI) and integrated discrimination index (IDI). *P*-value and 95% CI of NRI and IDI were estimated by 500 times bootstrap. The prognostic value of the new classifier was assessed by multivariable Cox proportional hazard models. Model 1 was adjusted for covariates, including sex, age, mean arterial blood pressure, and diabetes. Model 2 was additionally adjusted for eGFR. Hazard ratio (HR) and 95% CI was estimated for per SD increment in a classifier score or using the bottom quartile of a classifier score as a reference.

Bioinformatic analysis

To better interpret the underlying mechanisms between identified urinary peptides and CAD, the proteolysis processes that produced the polypeptides were considered. The potential proteases were predicted based on the N- and C-terminal cleavage sites of peptides by the Proteasix Knowledge Base (http://www.proteasix.org).[25] With the observed prediction mode, the cleavage sites were matched based on the literature. Subsequently, the parental proteins of the peptides, together with the predicted proteases, were submitted for pathway enrichment analysis to elucidate their biological functions. The pathway analysis was performed via the ClueGO plug-in (v 2.5.7) of Cytoscape v 3.7.2 using the Reactome pathway database (updated on 8 May 2020).[26,27] The minimum number of proteins to enrich a pathway was three and the minimum enrichment ratio was 4%. The significant threshold of *P*-values corrected by the Bonferroni step-down was 0.05. The clusters were based on pathway connectivity assessed by the predefined kappa score threshold of 0.4. And the cluster name was represented by the most significant pathway in a cluster.

Results

Participant characteristics

In the discovery cohort, the mean age was 58.4 [standard deviation (SD): 12.2] years and 27.8% were female. The baseline characteristics between individuals with and without CAD endpoints were similar in the discovery set ($P \geq 0.078$), except for eGFR (mean: 75.1 ± 17.0 vs. 83.1 ± 14.1, $P = 0.038$, *Table 1*). Of 893 participants in the validation cohort, the mean age was 52.7 (16.3) years, and 446 (49.9%) were women. Individuals who progressed to CAD endpoints tended to be male, older, having a history of hypertension, diabetes, chronic kidney disease, and on antihypertensive medication and aspirin compared with those without CAD endpoints ($P \leq 0.014$, *Table 2*).

Table 1 Participant characteristics in the discovery cohort

Characteristics	Discovery cohort			
	All ($n = 72$)	Controls ($n = 36$)	Case ($n = 36$)	*P*-value
Number with characteristic (%)				
Female	20 (27.8)	10 (27.8)	10 (27.8)	>0.99
Current smoking	17 (23.6)	6 (16.7)	11 (30.6)	0.27
Hypertension	46 (63.9)	23 (63.9)	23 (63.9)	>0.99
Treatment of hypertension	34 (47.2)	17 (47.2)	17 (47.2)	>0.99
Mean of characteristic (±SD)				
Age, years	58.4 ± 12.2	58.3 ± 12.2	58.6 ± 12.4	0.87
Body mass index, kg/m^2	27.4 ± 2.9	27.1 ± 3.0	27.7 ± 2.8	0.68
Systolic blood pressure, mmHg	133.1 ± 18.3	132.3 ± 17.5	133.8 ± 19.2	0.75
Diastolic blood pressure, mmHg	79.6 ± 10.4	79.3 ± 9.9	80.0 ± 11.1	0.96
Serum total cholesterol, mmol/L	5.54 ± 0.90	5.49 ± 0.86	5.58 ± 0.94	0.64
HDL cholesterol, mmol/L	1.26 ± 0.36	1.35 ± 0.39	1.18 ± 0.30	0.078
LDL cholesterol, mmol/L	3.44 ± 0.79	3.56 ± 0.67	3.33 ± 0.89	0.19
Blood glucose, mmol/L	5.36 ± 1.03	5.10 ± 0.58	5.62 ± 1.30	0.10
eGFR, mL/min/1.73m^2	79.1 ± 16.0	83.1 ± 14.1	75.1 ± 17.0	0.038

Current smoking refers to inhaling tobacco daily; hypertension was an office blood pressure of ≥140 mmHg systolic or ≥90 mmHg diastolic, or use of antihypertensive drugs.
eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, high-density lipoprotein; SD, standard deviation.

Urinary proteomic classifiers and the prediction of coronary artery disease

In the discovery cohort, a total of 518 sequenced peptides significantly differed between individuals with and without CAD after multiple testing corrections and permutation tests.

Subsequently, a urinary proteomic classifier integrating 160 significant peptides was developed in the discovery cohort using the SVM (regularization parameter: 64, kernel coefficient: 0.000256, epsilon: 0.001). When generalizing the new classifier to the independent validation cohort, this new classifier provided an AUC of 0.77 (95% CI: 0.70–0.84), 0.83 (95% CI: 0.76–0.86), and 0.82 (95% CI: 0.78–0.87) for the prediction of CAD endpoints at 3, 5, and 8 years, consistently outperforming to prior classifiers, CAD238 and ACSP75 (*Table 3*). Moreover, the addition of the new classifier significantly improved AUC on top of CAD238 (from 0.65–0.71 to 0.79–0.85) and ACSP75 (from 0.53–0.61 to 0.77–0.84). The combination of three classifiers yielded an increased AUC of 0.79 (0.73–0.85), 0.85 (0.78–0.87), and 0.84 (0.80–0.89) for the prediction of 3-, 5-, and 8-year CAD. Furthermore, the risk reclassification was improved by the addition of the new classifier on top of the Framingham risk score in 737 individuals from the FLEMENGHO study, as suggested by an NRI of 0.61 (95% CI: 0.25–0.95, $P = 0.001$) and IDI of 0.02 (95% CI: 0.02–0.05, $P = 0.39$). The risk reclassification improvement derived by the new classifier was also observed for the SCORE2 risk score with an NRI of 0.64 (95% CI: 0.28–0.98, $P = 0.001$) and IDI of 0.03 (95% CI: −0.003 to 0.06, $P = 0.097$).

Association between urinary proteomic classifier with coronary artery disease

The association between the new classifier and the risk of CAD is presented in *Table 4*. In the multivariable-adjusted model, a 1-SD increment in the new classifier was associated with a 1.62-fold (95% CI: 1.34–1.96) higher risk of incident CAD at 8-year follow-up. As shown in *Figure 2*, individuals with a higher new classifier score were at an increased risk of CAD. Compared with individuals in the bottom quartile of the new classifier, those in the highest quartile had a significantly higher risk of CAD (HR: 3.55, 95% CI: 1.84–6.85) after adjustment. When additionally adjusting for eGFR, slightly weakened associations that remained significant (HR: 1.54, 95% CI: 1.26–1.89 for 1-SD increment) were observed for the new classifier. After further adjusting for body mass index and smoking status (former/current/never), the new classifier was significantly associated with CAD (HR: 1.67, 95% CI: 1.11–2.53 for 1-SD increment) in 737 individuals from the FLEMENGHO study. Kaplan–Meier curves in *Figure 2* demonstrated that individuals at the top quartile of CAD238 or ACSP75 were at the highest risk of CAD compared with other quartiles. However, neither CAD238 nor ACSP75 was significantly associated with CAD after adjustment. Moreover, for 737 individuals from the FLEMENGHO study, the new classifier was positively associated with CAD, independent of the Framingham risk score (adjusted HR: 1.44, 95% CI: 1.01–2.95 for 1-SD increment) or the SCORE2 risk score (adjusted HR: 1.67, 95% CI: 1.14–2.45 for 1-SD increment).

Urinary peptides associated with coronary artery disease

These 160 urinary peptides were fragments of 58 parental proteins, mainly derived from collagens, such as collagen type I alpha I chain (36 peptides, 22.5%), collagen type III alpha chain (22 peptides, 13.8%), and collagen type I alpha II chain (9 peptides, 5.6%). Of 160 urinary peptides, most peptides were higher in individuals with CAD, except for 27 peptides from collagen type I alpha I chain (7 peptides), collagen type III alpha chain (5 peptides), uromodulin (6 peptides), apolipoprotein C-II (1 peptide), sarcalumenin (1 peptide), collagen type 1 alpha I (1 peptide), collagen type III alpha 1 (2 peptides), and collagen V alpha 2 (1 peptide). Supplementary material online, *Table S1* in the Supplementary material displays the list of 160 peptides, fold change in individuals with CAD, and their parental proteins. The elevated excretion of most collagen I and III fragments in individuals with CAD might suggest an upregulated collagen degradation. In addition to collagens, the excretions of other prominent proteins, including fibrinogen (10 peptides), uromodulin (7 peptides), polymeric immunoglobulin receptor (3 peptides), CD99 antigen (2 peptides), and insulin

Table 2 Participant characteristics in the validation cohort

Characteristics	Validation set ($n = 893$)			
	All ($n = 893$)	Controls ($n = 778$)	Case ($n = 115$)	*P*-value
Number with characteristic (%)				
Female	446 (49.9)	412 (53.0)	34 (29.6)	<0.0001
Current smoking[b]	161 (20.3)	145 (19.9)	16 (24.2)	0.42
Past smoking[a]	263 (35.7)	247 (35.2)	16 (45.7)	0.21
Hypertension	362 (40.5)	303 (39.0)	59 (51.3)	0.014
History of diabetes	117 (13.1)	65 (8.4)	52 (45.2)	<0.0001
Chronic kidney disease	129 (14.5)	77 (9.9)	52 (45.2)	<0.0001
Obesity[b]	166 (20.7)	146 (20.0)	20 (29.0)	0.087
Treatment of hypertension[a]	177 (24.0)	162 (23.1)	15 (42.9)	0.013
Aspirin[a]	68 (9.2)	55 (7.8)	13 (37.1)	<0.0001
Statins[a]	94 (12.8)	87 (12.4)	7 (20.0)	0.19
Mean of characteristic (±SD)				
Age, years	52.7 ± 16.3	50.8 ± 15.9	65.6 ± 12.9	<0.0001
BMI, kg/m^2[b]	26.7 ± 4.5	26.6 ± 4.5	28.3 ± 4.2	0.001
SBP, mmHg	130.4 ± 18.1	129.2 ± 17.5	138.4 ± 20.0	<0.0001
DBP, mmHg	79.2 ± 9.7	79.4 ± 9.6	77.9 ± 10.7	0.31
MAP, mmHg	109.8 ± 14.5	110.6 ± 14.0	104.2 ± 16.9	<0.0001
Total cholesterol, mmol/L[b]	5.17 (4.55–5.77)	5.17 (4.55–5.79)	5.20 (4.55–5.69)	0.83
HDL cholesterol, mmol/L[b]	1.40 (1.19–1.63)	1.42 (1.19–1.66)	1.23 (0.93–1.51)	<0.0001
LDL cholesterol, mmol/L[b]	3.08 (2.56–3.65)	3.08 (2.59–3.67)	3.13 (2.38–3.52)	0.23
Blood glucose, mmol/L[a]	4.90 ± 0.76	4.86 ± 0.60	5.72 ± 2.11	<0.0001
SCORE2 risk score[a]	2.64 (1.02–5.46)	2.49 (0.95–4.93)	9.06 (5.64–13.88)	<0.0001
Framingham risk score[a]	7.10 (2.42–14.91)	6.50 (2.24–13.96)	26.42 (16.05–47.37)	<0.0001
eGFR, mL/min/1.73 m^2	81.1 ± 22.6	83.9 ± 20.8	62.1 ± 25.3	<0.0001

Hypertension was an office blood pressure of ≥140 mmHg systolic or ≥90 mmHg diastolic, or use of antihypertensive drugs. Chronic kidney disease was defined as eGFR <60 mL/min/1.73 m^2.

BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, high-density lipoprotein; MAP, mean arterial blood pressure; SBP, systolic blood pressure; SD, standard deviation.

[a]Clinical variables were available for 737 participants from the FLEMENGHO study.

[b]Available clinical variables: current smoking ($n = 795$), obesity ($n = 801$), BMI ($n = 801$), total cholesterol ($n = 765$), HDL cholesterol ($n = 791$), and LDL cholesterol ($n = 786$).

(2 peptides), and insulin-like growth factors II were upregulated in participants with CAD. The remaining parental proteins functioned diversely, including lipid metabolism (apolipoprotein C-II, apolipoprotein C-III, apolipoprotein L1, and clusterin), proliferation (thymosin beta-4), tissue remodelling (clusterin), immune response (complement C4-A and complement factor B), and inflammation (interleukin-1 receptor antagonist protein).

Bioinformatics was performed to obtain a comprehensive overview of the proteases that could lead to the generation of peptides associated with CAD. According to the cleavage sites, 11 proteases were predicted to participate in the fragmentation of proteins associated with CAD. Peptides that were higher in individuals with CAD predicted 11 upregulated matrix metalloproteinases (MMPs) that mainly involve in collagen degradation, including MMP 1, 2, 8, 9, 13, 14, cathepsin K, and neuroendocrine convertase 1. Downregulated peptides predicted lower activity of MMP 2 and 9 as well (see Supplementary material online, *Table S2* in the Supplementary material).

The parental proteins of urinary peptides and their upstream proteases might be involved in the pathogenesis of CAD. Pathway enrichment analysis mapped 8 clusters consisting of 41 pathways (*Figure 3* and Supplementary material online, *Table S3* in the Supplementary material). The major pathways enriched were extracellular matrix turnover and signalling, cell surface interactions, plasma lipoprotein metabolism, activation of MMP, complement cascade, proliferation, and insulin processing.

Discussion

In the present study, we developed a novel urinary proteomic classifier associated with CAD using a machine learning approach and validated its predictive performance in an independent prospective cohort of 893 individuals. Specifically, the novel proteomic classifier outperformed previously developed CAD-related classifiers in terms of the prediction of CAD endpoints, and the addition of the novel proteomic classifier to previous classifiers significantly improved its predictive performance, with the highest AUC of 0.87. Moreover, using multivariable Cox regression models, the novel proteomic classifier was significantly associated with the risk of reaching an ischaemic endpoint at 8-year follow up. The novel proteomic classifier was comprised of 160 urinary polypeptides that may provide mechanistic insights into CAD and suggest targeted interventions to halt or reverse the development and progression of ischaemic heart disease.

It is challenging to diagnose and intervene in CAD at an early stage, as CAD is characterized by coronary artery atherosclerosis that can progress asymptomatically for extended periods of time. Therefore,

prognostic biomarkers are important to stratify CAD risk and identify individuals with high risk who might benefit from early interventions. Evidence-based CAD risk prediction algorithms and intervention strategies have been established and recommended by the European Society of Cardiology and the American Heart Association.[28,29] However, the high morbidity and mortality caused by CAD persist. Therefore, identifying potential markers and incorporating them into the current risk prediction model is advocated by the European Society of Cardiology.[28] Previous studies have shown that urinary proteomics is a promising tool to detect CAD. Two urinary proteomic panels were successively developed, and they presented good performance (sensitivity and specificity > 80%) for the screening of CAD in limited samples.[30,31] However, these two proteomic panels failed to maintain their performance after generalizing to a cohort with 138 individuals.[10] Following this, a urinary proteomic pattern with 238 polypeptides, called CAD238, was developed using a machine learning approach, and its diagnostic performance and association with CAD risk were subsequently verified.[10,11] Moreover, a urinary proteomic classifier, ACSP75, was constructed specifically for the prediction of acute coronary syndromes, although its performance (AUC = 0.64) needs to be improved.[13]

While these previous findings clearly support the concept and utilization of urinary proteome analysis for CAD-specific biomarker discovery, we set out to further improve its predictive performance in a relatively large and well-characterized cohort. Compared with previous studies on urinary proteomics, there were several distinct features of the present study. Given the asymptomatic nature of early CAD, we designed a prospective study, which was more likely to detect subtle pathological biomarkers before proven CAD. To comprehensively examine the generalizability of developed biomarkers, individuals with various clinical contexts in the validation cohort hence were at diverse risk profiles, including the general population (low risk) and patients with diabetes (high risk). Specifically, integrating the new classifier into conventional cardiovascular risk prediction algorithms (the Framingham and SCORE2 risk scores) can further improve risk reclassification, which implies that the new proteomic classifier might be a promising tool for CAD risk prediction.

Despite the advances in urinary proteomic biomarker research in the context of CAD, several hurdles remain. First, it is unclear whether the novel urinary proteomic classifier could distinguish the subtypes of

Table 3 Predictive performance of urinary proteomic markers for CAD

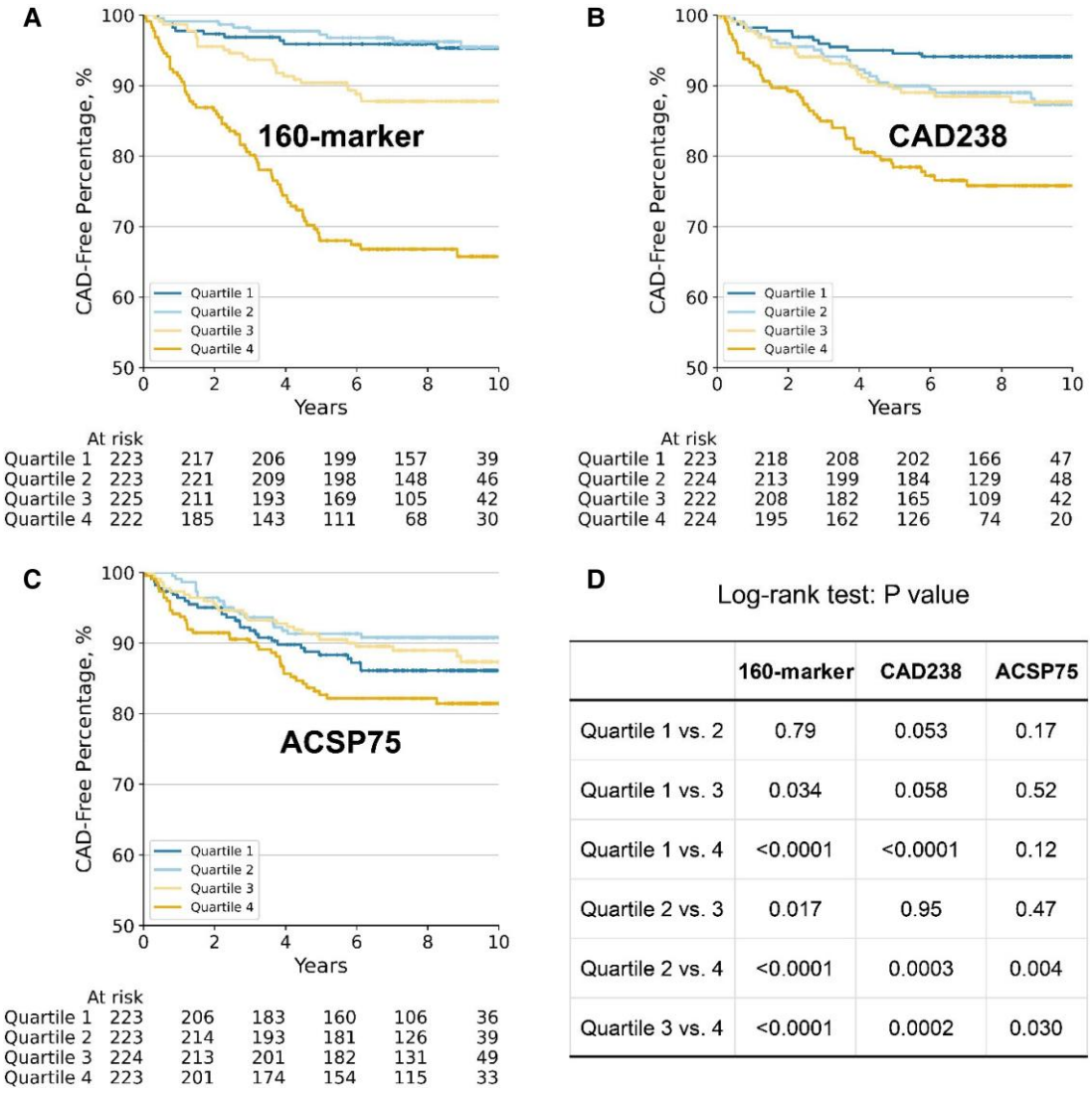
	Time-dependent AUC (95% CI)		
No. of events/at risk	**3-year 69/784**	**5-year 102/716**	**8-year 111/478**
160-marker	0.77 (0.70–0.84)	0.83 (0.76–0.86)	0.82 (0.78–0.87)
CAD238	0.65 (0.58–0.72)	0.66 (0.61–0.73)	0.71 (0.66–0.77)
ACSP75	0.53 (0.45–0.61)	0.61 (0.48–0.64)	0.53 (0.47–0.60)
160-marker + CAD238	0.79 (0.72–0.86)[a]	0.84 (0.77–0.88)[a]	0.85 (0.80–0.89)[a]
160-marker + ACSP75	0.77 (0.71–0.84)[b]	0.84 (0.77–0.86)[b]	0.82 (0.78–0.86)[b]
160-marker + CAD238 + ACSP75	0.79 (0.73–0.85)	0.85 (0.78–0.87)	0.84 (0.80–0.89)[c]

[a]AUC was significantly improved ($P < 0.05$) compared with the model with CAD238.
[b]AUC was significantly improved ($P < 0.05$) compared with the model with ACSP75.
[c]AUC was significantly improved ($P < 0.05$) compared with the model with 160-marker.

Table 4 Risk of CAD by baseline urinary proteomic classifier

	Events/at risk	Multivariable model 1		Multivariable model 2	
		HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
160-marker					
Per 1-SD increment	115/893	1.62 (1.34–1.96)	<0.0001	1.54 (1.26–1.89)	<0.0001
Quartile 1	11/223	Reference		Reference	
Quartile 2	9/223	0.89 (0.37–2.17)	0.80	0.88 (0.36–2.14)	0.78
Quartile 3	26/225	2.27 (1.11–4.62)	0.024	2.14 (1.05–4.38)	0.037
Quartile 4	69/222	3.55 (1.84–6.85)	0.0002	3.19 (1.63–6.27)	0.0007
CAD238					
Per 1-SD increment	115/893	1.03 (0.86–1.22)	0.78	1.01 (0.85–1.21)	0.90
Quartile 1	13/223	Reference		Reference	
Quartile 2	27/224	2.35 (1.21–4.57)	0.012	2.25 (1.16–4.39)	0.017
Quartile 3	25/222	1.35 (0.68–2.66)	0.39	1.19 (0.60–2.38)	0.61
Quartile 4	50/224	1.64 (0.85–3.15)	0.14	1.47 (0.76–2.84)	0.25
ACSP75					
Per 1-SD increment	115/893	1.05 (0.87–1.28)	0.61	1.09 (0.90–1.32)	0.38
Quartile 1	29/223	Reference		Reference	
Quartile 2	20/223	0.51 (0.29–0.90)	0.021	0.55 (0.31–0.97)	0.040
Quartile 3	26/224	0.76 (0.44–1.29)	0.30	0.79 (0.46–1.35)	0.39
Quartile 4	40/223	0.98 (0.61–1.59)	0.94	1.09 (0.67–1.77)	0.74

Model 1 was adjusted for sex, age, mean arterial blood pressure, and history of diabetes. Model 2 was adjusted as for model 1 and for eGFR.



	160-marker	CAD238	ACSP75
Quartile 1 vs. 2	0.79	0.053	0.17
Quartile 1 vs. 3	0.034	0.058	0.52
Quartile 1 vs. 4	<0.0001	<0.0001	0.12
Quartile 2 vs. 3	0.017	0.95	0.47
Quartile 2 vs. 4	<0.0001	0.0003	0.004
Quartile 3 vs. 4	<0.0001	0.0002	0.030

Figure 2 Kaplan–Meier curves for coronary artery disease according to the quartiles of urinary proteomic classifiers. The top quartile (Quartile 4) of the urinary proteomic classifiers was associated with an increased risk of coronary artery disease. (*A*) 160-marker. (*B*) CAD238. (*C*) ACSP75. (*D*) *P*-values for strata comparison. CAD, coronary artery disease.

CAD, such as acute myocardial infarction, angina, and cardiac arrest. The risk assessment of CAD subtypes would stimulate individualized prevention and treatment strategies that would be more effective than a one-size-fits-all strategy. The existing biomarkers were specifically developed to detect composite CAD endpoints or a single subtype, such as ACSP75. Second, the developed CAD-specific biomarkers require external validation, especially in cohorts with distinct comorbidities. Oellgaard *et al.*[32] reported that ACSP75 was associated with cardiovascular events, but CAD238 did not present a significant prognostic association in patients with diabetes. Although this novel proteomic classifier showed prognostic potential for individuals, including diabetes patients, large, multicentre prospective studies may help to resolve these challenges.

Progress in genetic 'omics' techniques coupled with advanced machine learning provides opportunities to identify novel biomarkers that can enhance CAD prediction beyond conventional risk algorithms.[33–36] In the past decades, polygenic risk scores integrating various genetic variants have been extensively investigated to predict the genetic risk of CAD that is responsible for approximately 30–40% of the heritability of CAD.[33,34] Unlike clinical risk scores that are more applicable for CAD prediction in adulthood, polygenic risk scores can estimate the lifelong risk at an early age.[33,34,36] In comparison with polygenic risk scores, proteomics is influenced by various factors such as genetics, behaviours, and environmental factors, thereby reflecting comprehensive changes in CAD risk. Prior studies have demonstrated that plasma protein–based risk scores can improve CAD prediction on top of clinical risk factors, for both primary and secondary prevention scenarios.[37–39] Similarly, the plasma proteins that contributed to the risk prediction scores also underscored the importance of matrix degradation, apoptosis, and inflammation in the development of atherosclerosis.[37,38] The advances in genetic and proteomic approaches for cardiovascular risk prediction are noteworthy, yet it is imperative that future studies evaluate their utilization in the management of CAD and cost-effectiveness to facilitate clinical implementation.

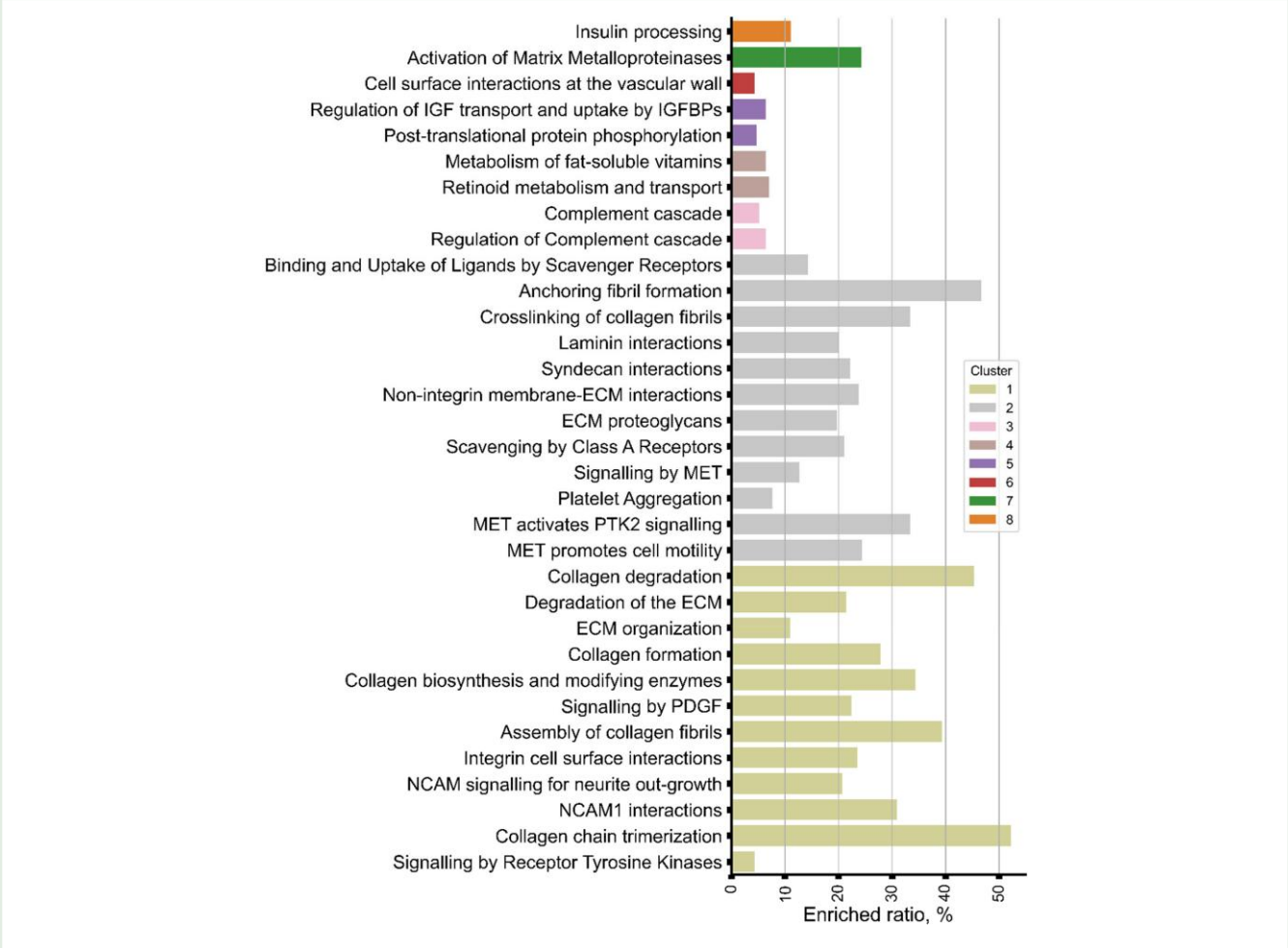


Figure 3 Pathway enrichment analysis based on the parental proteins and predicted proteases of 160 urinary peptides. Enriched pathways involved in seven clusters: extracellular matrix turnover and signalling (cluster 1), cell surface interactions at the vascular wall (cluster 2), plasma lipoprotein assembly, remodelling, and clearance (cluster 3), activation of matrix metalloproteinases (cluster 4), complement cascade (cluster 5), regulation of insulin-like growth factor transport and uptake by insulin-like growth factor binding proteins (cluster 6), and insulin processing (cluster 7). All displayed pathways had $P < 0.05$. Enrichment ratio represents the number of submitted proteins in a particular pathway to the total number of proteins in the pathway. ECM, extracellular matrix; LPL, lipoprotein lipase; LIPC, hepatic lipase; MET, receptor tyrosine kinase; NCAM1, neural cell adhesion molecule; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTK, protein tyrosine kinase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein.

Endogenous peptides and small proteins in urine provide clues to the status of their larger precursor proteins, including the degradation process and posttranslational modifications.[6] These subtle clues can help to puzzle out complex pathological mechanisms. The pathogenesis of CAD is complex and multifactorial, caused by diverse mechanisms. The complex nature of CAD that is hardly reflected by a single molecule requires a comprehensive search and integration of biomarkers. Previous urinary proteomic panels proposed by Zimmerli *et al.*[30] and von Zur Muhlen *et al.*[31] are comprised mainly of collagen I and collagen type III fragments. The constitution of CAD238 and ACSP75 is more diverse, involving collagen, fibrinogen, and mucin.[10,13] It appears that collagen turnover in the extracellular matrix is disproportionally regulated in individuals prior to their CAD events. Collagen type I contributes to 60% of protein contents in the atherosclerotic plaque and is an essential component of the fibrous cap.[40] Upregulated degradation of collagen type I can lead to a thinner fibrous cap, a trigger for atherosclerotic plaque rupture.[41] Circulating MMP-mediated collagen type I biomarker has been suggested to be positively associated with a higher risk of cardiovascular events.[40] Our study suggests that the majority of collagen type I and III fragments was more abundantly excreted in urine, prior to future CAD events, indicating an upregulated collagen degradation in extracellular matrix. This was consistent with the predicted upregulated MMPs, such as MMP 1, 2, 8, 9, 13, 14, and cathepsin K.

The overlap between our proteomic characterization, CAD238, and ACSP75 also included uromodulin. Uromodulin, produced by renal epithelial cells, is considered the most abundant glycoprotein in urine and can be found in the blood as well.[42] Uromodulin is frequently associated with the risk of chronic kidney disease and hypertension.[42,43] Recent evidence demonstrated a reverse association of serum uromodulin with coronary artery events and coronary artery calcification, even though the mechanism is unclear.[44,45] Similarly, we also observed that the level of urinary uromodulin peptides was lower in individuals who progressed to CAD.

There were several unique proteins included in the novel proteomic classifier. For instance, clusterin is a glycoprotein, also known as apolipoprotein J that plays an essential role in inflammation, lipid metabolism, and atherosclerosis.[46] High serum clusterin is suggested to be associated with premature CAD.[47] Consistent with this finding, we also observed a higher urinary clusterin peptide for individuals with CAD endpoints. Clusterin can stimulate vascular smooth muscle cell proliferation and migration and the expression of TNF-α and early growth response 1 in the atherosclerotic lesion, which further contributes to the pathogenesis of atherosclerosis.[48] On the other hand, a previous study suggested that upregulated clusterin can be antiatherogenic by reducing the production of reactive oxygen species (ROS) and proinflammatory factors.[46] Carbonic anhydrase I is an enzyme that catalyses the reversible hydration of carbon dioxide, and it links to atherosclerotic calcification and the progression of atherosclerosis.[49] In addition to calcium carbonate formation, it regulates the carboxylation of matrix Gla protein which is an essential inhibitor of vascular calcification contributing to the typical pattern of atherosclerosis.[50] Carbonic anhydrase stimulates primary metabolic processes, such as carbon dioxide and acid base balance, that involve in the initiation of atherosclerosis formation.[51] Folate receptor alpha CD99 is another significantly downregulated protein in our study. CD99, a cell adhesion molecule, is expressed by endothelial cells and engages in the recruitment of monocytes and lymphocytes. The recruited inflammatory cells can migrate to atherosclerotic regions, bind oxidated lipoprotein particles, and become arterial foam cells, secrete proinflammatory cytokines, initial inflammation, and stimulate the production of ROS.[52] Vaccination against CD99 can effectively attenuate the progression of atherosclerotic plaques by lowering leukocytes in atherosclerotic lesions.[53]

Strengths and limitations

Our study has several strengths, including systematic urinary proteome analyses with good reproducibility, validation of the urinary proteomic in an independent, relatively large cohort, and assessment of the prognostic value. There are several limitations of our study. First, the association between the urinary proteomic classifier and the severity or subtypes of coronary events was not investigated, which requires a large, prospective study. Second, future studies are warranted to further evaluate whether the urinary proteomic classifier can identify plaque volume and composition measured by ultrasound or computerized tomography. Correlating urinary biomarkers with subclinical lesions could help to determine when interventions, such as statin therapy, should be initiated. Last, pathway analysis of these peptides relied on existing studies; thus, their roles in the development of CAD might be different, which needs to be determined in preclinical experimental and epidemiological studies.

Conclusions

This study developed and validated a urinary proteomic classifier for the prediction of ischaemic CAD endpoint with good performance. The peptides constituting the proteomic classifier were involved in diverse pathways associated with atherosclerosis, including collagen turnovers, lipid metabolism, and inflammation.

Supplementary material

Supplementary material is available at *European Journal of Preventive Cardiology*.

Acknowledgements

The authors gratefully acknowledge the contribution of Prof Harald Mischak and Agnieszka Latosinska from Mosaiques Diagnostics (Hannover, Germany) for urinary proteomic profiling, data analyses, and interpretation and the clerical contribution of Renilde Wolfs.

Author contributions

D.W. and Z.-Y.Z. conceptualized and designed the study. Z.-Y.Z. contributed to data acquisition. D.W and Z.-Y.Z. performed analysis. D.W. initially drafted the manuscript. All authors interpreted the data and critically revised the manuscript. All authors reviewed and approved the final manuscript.

Funding

The European Research Area Net for Cardiovascular Diseases Funding (JTC2017-046-PROACT) and KU Leuven Funding (STG-18-00379) currently support this study.

Conflict of interest: The authors have no conflicts of interest to declare.

Data availability

The data underlying this article cannot be shared publicly due to the privacy of the participants. The data can only be reasonably requested to the corresponding author.

References

1. Dai H, Much AA, Maor E, Asher E, Younis A, Xu Y, *et al.* Global, regional, and national burden of ischaemic heart disease and its attributable risk factors, 1990–2017: results from the Global Burden of Disease Study 2017. *Eur Heart J Qual Care Clin Outcomes* 2022;**8**:50–60.
2. Matsuura Y, Kanter JE, Bornfeldt KE. Highlighting residual atherosclerotic cardiovascular disease risk. *Arterioscler Thromb Vasc Biol* 2019;**39**:e1–e9.
3. Lam MP, Ping P, Murphy E. Proteomics research in cardiovascular medicine and biomarker discovery. *J Am Coll Cardiol* 2016;**68**:2819–2830.
4. Latosinska A, Siwy J, Faguer S, Beige J, Mischak H, Schanstra JP. Value of urine peptides in assessing kidney and cardiovascular disease. *Proteomics Clin Appl* 2021;**15**:e2000027.
5. Julian BA, Suzuki H, Suzuki Y, Tomino Y, Spasovski G, Novak J. Sources of urinary proteins and their analysis by urinary proteomics for the detection of biomarkers of disease. *Proteomics Clin Appl* 2009;**3**:1029–1043.
6. Wei D, Melgarejo JD, Thijs L, Temmerman X, Vanassche T, Van Aelst L, *et al.* Urinary proteomic profile of arterial stiffness is associated with mortality and cardiovascular outcomes. *J Am Heart Assoc* 2022;**11**:e024769.
7. Wei D, Melgarejo J, Vanassche T, Van Aelst L, Janssens S, Verhamme P, *et al.* Urinary matrix Gla protein is associated with mortality risk in Flemish population: a prospective study. *Front Cardiovasc Med* 2022;**9**:894447.
8. Zhang ZY, Ravassa S, Nkuipou-Kenfack E, Yang WY, Kerr SM, Koeck T, *et al.* Novel urinary peptidomic classifier predicts incident heart failure. *J Am Heart Assoc* 2017;**6**:e005432.
9. He T, Mischak M, Clark AL, Campbell RT, Delles C, Diez J, *et al.* Urinary peptides in heart failure: a link to molecular pathophysiology. *Eur J Heart Fail* 2021;**23**:1875–1887.
10. Delles C, Schiffer E, von Zur Muhlen C, Peter K, Rossing P, Parving HH, *et al.* Urinary proteomic diagnosis of coronary artery disease: identification and clinical validation in 623 individuals. *J Hypertens* 2010;**28**:2316–2322.
11. Brown CE, McCarthy NS, Hughes AD, Sever P, Stalmach A, Mullen W, *et al.* Urinary proteomic biomarkers to predict cardiovascular events. *Proteomics Clin Appl* 2015;**9**:610–617.
12. Neisius U, Koeck T, Mischak H, Rossi SH, Olson E, Carty DM, *et al.* Urine proteomics in the diagnosis of stable angina. *BMC Cardiovasc Disord* 2016;**16**:70.
13. Htun NM, Magliano DJ, Zhang ZY, Lyons J, Petit T, Nkuipou-Kenfack E, *et al.* Prediction of acute coronary syndromes by urinary proteome analysis. *PLoS One* 2017;**12**:e0172036.
14. Latosinska A, Siwy J, Mischak H, Frantzi M. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis* 2019;**40**:2294–2308.
15. Dabelea D, Kinney G, Snell-Bergeon JK, Hokanson JE, Eckel RH, Ehrlich J, *et al.* Effect of type 1 diabetes on the gender difference in coronary artery calcification: a role for insulin resistance? The Coronary Artery Calcification in Type 1 Diabetes (CACTI) Study. *Diabetes* 2003;**52**:2833–2839.

16. Snell-Bergeon JK, Maahs DM, Ogden LG, Kinney GL, Hokanson JE, Schiffer E, *et al.* Evaluation of urinary biomarkers for coronary artery disease, diabetes, and diabetic kidney disease. *Diabetes Technol Ther* 2009;**11**:1–9.
17. Verbeke F, Siwy J, Van Biesen W, Mischak H, Pletinck A, Schepers E, *et al.* The urinary proteomics classifier chronic kidney disease 273 predicts cardiovascular outcome in patients with chronic kidney disease. *Nephrol Dial Transplant* 2021;**36**:811–818.
18. Barr EL, Zimmet PZ, Welborn TA, Jolley D, Magliano DJ, Dunstan DW, *et al.* Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;**116**:151–157.
19. Alkhalaf A, Zurbig P, Bakker SJ, Bilo HJ, Cerna M, Fischer C, *et al.* Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy. *PLoS One* 2010;**5**: e13421.
20. Jantos-Siwy J, Schiffer E, Brand K, Schumann G, Rossing K, Delles C, *et al.* Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res* 2009;**8**:268–281.
21. Mischak H, Kolch W, Aivaliotis M, Bouyssie D, Court M, Dihazi H, *et al.* Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. *Proteomics Clin Appl* 2010;**4**:464–478.
22. Mischak H, Vlahou A, Ioannidis JP. Technical aspects and inter-laboratory variability in native peptide profiling: the CE-MS experience. *Clin Biochem* 2013;**46**:432–443.
23. Frantzi M, Metzger J, Banks RE, Husi H, Klein J, Dakna M, *et al.* Discovery and validation of urinary biomarkers for detection of renal cell carcinoma. *J Proteomics* 2014;**98**:44–58.
24. D'Agostino RB S, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, *et al.* General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation* 2008;**117**:743–753.
25. Klein J, Eales J, Zurbig P, Vlahou A, Mischak H, Stevens R. Proteasix: a tool for automated and large-scale prediction of proteases involved in naturally occurring peptide generation. *Proteomics* 2013;**13**:1077–1082.
26. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, *et al.* ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 2009;**25**:1091–1093.
27. Fabregat A, Sidiropoulos K, Viteri G, Forner O, Marin-Garcia P, Arnau V, *et al.* Reactome pathway analysis: a high-performance in-memory approach. *BMC Bioinformatics* 2017; **18**:142.
28. Visseren FLJ, Mach F, Smulders YM, Carballo D, Koskinas KC, Back M, *et al.* ESC guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J* 2021;**2021**: 3227–3337.
29. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, *et al.* ACC/AHA guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines. *Circulation* 2019;**2019**:e596–e646.
30. Zimmerli LU, Schiffer E, Zurbig P, Good DM, Kellmann M, Mouls L, *et al.* Urinary proteomic biomarkers in coronary artery disease. *Mol Cell Proteomics* 2008;**7**:290–298.
31. von Zur Muhlen C, Schiffer E, Zuerbig P, Kellmann M, Brasse M, Meert N, *et al.* Evaluation of urine proteome pattern analysis for its potential to reflect coronary artery atherosclerosis in symptomatic patients. *J Proteome Res* 2009;**8**:335–345.
32. Oellgaard J, Gaede P, Persson F, Rossing P, Parving HH, Pedersen O. Application of urinary proteomics as possible risk predictor of renal and cardiovascular complications in patients with type 2-diabetes and microalbuminuria. *J Diabetes Complications* 2018;**32**: 1133–1140.
33. Figtree GA, Vernon ST, Nicholls SJ. Taking the next steps to implement polygenic risk scoring for improved risk stratification and primary prevention of coronary artery disease. *Eur J Prev Cardiol* 2022;**29**:580–587.
34. Levin MG, Rader DJ. Polygenic risk scores and coronary artery disease: ready for prime time? *Circulation* 2020;**141**:637–640.
35. Lindsey ML, Mayr M, Gomes AV, Delles C, Arrell DK, Murphy AM, *et al.* Transformative impact of proteomics on cardiovascular health and disease: a scientific statement from the American Heart Association. *Circulation* 2015;**132**:852–872.
36. O'Sullivan JW, Raghavan S, Marquez-Luna C, Luzum JA, Damrauer SM, Ashley EA, *et al.* Polygenic risk scores for cardiovascular disease: a scientific statement from the American Heart Association. *Circulation* 2022;**146**:e93–e118.
37. Hoogeveen RM, Pereira JPB, Nurmohamed NS, Zampoleri V, Bom MJ, Baragetti A, *et al.* Improved cardiovascular risk prediction using targeted plasma proteomics in primary prevention. *Eur Heart J* 2020;**41**:3998–4007.
38. Nurmohamed NS, Belo Pereira JP, Hoogeveen RM, Kroon J, Kraaijenhof JM, Waissi F, *et al.* Targeted proteomics improves cardiovascular risk prediction in secondary prevention. *Eur Heart J* 2022;**43**:1569–1577.
39. Ganz P, Heidecker B, Hveem K, Jonasson C, Kato S, Segal MR, *et al.* Development and validation of a protein-based risk score for cardiovascular outcomes among patients with stable coronary heart disease. *JAMA* 2016;**315**:2532–2541.
40. Nielsen S H, Tengryd C, Edsfeldt A, Brix S, Genovese F, Bengtsson E, *et al.* A biomarker of collagen type I degradation is associated with cardiovascular events and mortality in patients with atherosclerosis. *J Intern Med* 2019;**285**:118–123.
41. Lutgens E, van Suylen RJ, Faber BC, Gijbels MJ, Eurlings PM, Bijnens AP, *et al.* Atherosclerotic plaque rupture: local or systemic process? *Arterioscler Thromb Vasc Biol* 2003;**23**:2123–2130.
42. Devuyst O, Olinger E, Rampoldi L. Uromodulin: from physiology to rare and complex kidney disorders. *Nat Rev Nephrol* 2017;**13**:525–544.
43. Padmanabhan S, Graham L, Ferreri NR, Graham D, McBride M, Dominiczak AF. Uromodulin, an emerging novel pathway for blood pressure regulation and hypertension. *Hypertension* 2014;**64**:918–923.
44. Leiherer A, Muendlein A, Saely CH, Ebner J, Brandtner EM, Fraunberger P, *et al.* Serum uromodulin is a predictive biomarker for cardiovascular events and overall mortality in coronary patients. *Int J Cardiol* 2017;**231**:6–12.
45. Bjornstad P, Wiromrat P, Johnson RJ, Sippl R, Cherney DZI, Wong R, *et al.* Serum uromodulin predicts less coronary artery calcification and diabetic kidney disease over 12 years in adults with type 1 diabetes: the CACTI study. *Diabetes Care* 2019;**42**:297–302.
46. Park S, Mathis KW, Lee IK. The physiological roles of apolipoprotein J/clusterin in metabolic and cardiovascular diseases. *Rev Endocr Metab Disord* 2014;**15**:45–53.
47. Zhu H, Liu M, Zhai T, Pan H, Wang L, Yang H, *et al.* High serum clusterin levels are associated with premature coronary artery disease in a Chinese population. *Diabetes Metab Res Rev* 2019;**35**:e3128.
48. Hamada N, Miyata M, Eto H, Ikeda Y, Shirasawa T, Akasaki Y, *et al.* Loss of clusterin limits atherosclerosis in apolipoprotein E-deficient mice via reduced expression of Egr-1 and TNF-alpha. *J Atheroscler Thromb* 2011;**18**:209–216.
49. Yuan L, Wang M, Liu T, Lei Y, Miao Q, Li Q, *et al.* Carbonic anhydrase 1-mediated calcification is associated with atherosclerosis, and methazolamide alleviates its pathogenesis. *Front Pharmacol* 2019;**10**:766.
50. Adeva-Andany MM, Fernandez-Fernandez C, Sanchez-Bello R, Donapetry-Garcia C, Martinez-Rodriguez J. The role of carbonic anhydrase in the pathogenesis of vascular calcification in humans. *Atherosclerosis* 2015;**241**:183–191.
51. Gamble W. Atherosclerosis: the carbonic anhydrase, carbon dioxide, calcium concerted theory. *J Theor Biol* 2006;**239**:16–21.
52. Libby P. Inflammation in atherosclerosis. *Nature* 2002;**420**:868–874.
53. van Wanrooij EJ, de Vos P, Bixel MG, Vestweber D, van Berkel TJ, Kuiper J. Vaccination against CD99 inhibits atherogenesis in low-density lipoprotein receptor-deficient mice. *Cardiovasc Res* 2008;**78**:590–596.