Urinary proteomic biomarkers to predict cardiovascular events

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Abstract

Purpose We have previously demonstrated associations between the urinary proteome profile and coronary artery disease (CAD) in cross-sectional studies. Here, we evaluate the potential of a urinary proteomic panel as a predictor of CAD in the hypertensive atherosclerotic cardiovascular disease (HACVD) substudy population of the Anglo-Scandinavian Cardiac Outcomes Trial study.

Experimental design Thirty-seven cases with primary CAD endpoint were matched for sex and age to controls who had not reached a CAD endpoint during the study. Spot urine samples were analyzed using CE coupled to Micro-TOF MS. A previously developed 238-marker CE-MS model for diagnosis of CAD (CAD$_{238}$) was assessed for its predictive potential.

Results Sixty urine samples (32 cases; 28 controls; 88% male, mean age 64 ± 5 years) were analyzed. There was a trend toward healthier values in controls for the CAD model classifier (−0.432 ± 0.326 versus −0.587 ± 0.297, p = 0.170), and the CAD model showed statistical significance on Kaplan–Meier survival analysis p = 0.021. We found 190 individual markers out of 1501 urinary peptides that separated cases and controls (AUC >0.6). Of these, 25 peptides were also components of CAD$_{238}$.

Conclusion and clinical relevance A urinary proteome panel originally developed in a cross-sectional study predicts CAD endpoints independent of age and sex in a well-controlled prospective study.

Abbreviations

ACE angiotensin-converting enzyme
ASCOT Anglo-Scandinavian Cardiac Outcomes Trial
CAD coronary artery disease
CKD chronic kidney disease
HACVD hypertensive atherosclerotic cardiovascular disease
1 Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the United Kingdom and worldwide 1, 2. Cardiovascular risk assessment and risk prediction are performed daily in primary and secondary care with traditional scoring methods combining classical risk factors such as age, sex, smoking, blood pressure, and cholesterol. These methods calculate average percentage likelihood of a patient suffering a cardiovascular event (acute coronary syndrome, stroke, or transient ischaemic attack) 3-5 and assist the clinician in guiding initiation and intensity of therapies 4. However, such individual risk assessments are not always precise. The main challenge of cardiovascular risk stratification currently lies with patients who are classified as being at intermediate risk by conventional methods 6. More refined methods of risk assessment, especially for those at intermediate risk, are therefore required.

Clinical Relevance

Individualized cardiovascular risk prediction remains an unmet clinical need in patients with hypertension and other risk factors. Studying multiple biomarkers at a time, proteomics has the potential to detect subclinical disease and thereby improve risk stratification and targeted treatment. In the present study, we have taken our clinical proteomic studies into coronary artery disease one step further, from a cross-sectional study to prediction, by evaluating the potential of a panel of urinary polypeptides to predict future coronary events.

Established cardiovascular disease is preceded by an asymptomatic phase 7 and the identification of precursors of cardiovascular disease may allow the detection of damage at an early and potentially reversible stage 8. Biomarkers are an example of one such tool to better identify high-risk individuals, to diagnose disease conditions promptly and accurately, and to provide a prognosis and treat patients effectively 2.

Most available biomarkers have been developed as an extension of targeted physiological studies, investigating known pathways. By contrast, emerging technologies are beginning to allow the systematic, unbiased characterization of variation in proteins associated with disease conditions 9. Urine is a promising medium for proteomic-based research as it is easily accessible and stable when frozen without requiring special preparation 10, 11. We have previously reported and validated the use of urinary proteomics in the diagnosis of coronary artery disease (CAD) 12. Urinary proteomic biomarkers have also been studied in other conditions such as preeclampsia 10, diabetes 13, diabetic nephropathy 14,
stroke 15, and heart failure 16. We hypothesized that a CAD-specific urinary polypeptide panel not only predicts the presence or absence of disease, but also, critically, future cardiovascular events, an observation that would extend current cross-sectional studies. In order to address this question we used urine samples available from the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT), an investigator-led, multicenter, randomized trial to compare treatment strategies for the prevention of CAD in patients with hypertension who had multiple risk factors (see below for inclusion and exclusion criteria) 17.

2 Materials and methods

2.1 Patients

The aim of ASCOT was to determine the answers to several questions regarding the management of hypertension, particularly whether combination therapy with a dihydropyridine calcium channel blocker and angiotensin-converting enzyme (ACE) inhibitor produced greater benefits in terms of reducing coronary heart disease events than the standard beta-blocker and diuretic combination 17. It also sought to determine whether lipid lowering with a statin provided additional benefit in those hypertensive patients with average or below average levels of serum total cholesterol. Recruitment ran from February 1998 to May 2000 by which time 19,342 hypertensive patients had been randomized to the two antihypertensive treatment regimes. Methods and study design of ASCOT have been described in detail elsewhere 18.

Randomization was to either a standard antihypertensive regimen (β-blocker + diuretic) or to a more contemporary regimen (calcium antagonist + angiotensin converting enzyme inhibitor). Patients were ineligible if they had any of the following: previous myocardial infarction, currently treated angina, a cerebrovascular event within the previous 3 months, congestive heart failure, uncontrolled arrhythmias, or any major noncardiovascular disease. In addition, the benefits of cholesterol lowering in the primary prevention of coronary heart disease in hypertensive patients were assessed in a lipid-lowering arm of this trial (ASCOT-LLA). A total of 10,350 patients with a nonfasting cholesterol level of ≤ 6.5 mmol/L were recruited into ASCOT-LLA, and randomized to either atorvastatin 10 mg or placebo 19.

The population examined in this study was the hypertension associated cardiovascular disease (HACVD) cohort; an intensively phenotyped substudy of (ASCOT) 20. The HACVD substudy involved intensive phenotyping of 1395
subjects, along with DNA collection, with the intention of identifying unrecognized genetic influences on the development of hypertension-associated cardiovascular disease. Baseline demographic data (age, gender, and ethnicity), levels of cardiovascular risk factors (cigarette smoking, diabetes, dyslipidaemia, blood pressure, and body mass index), evidence of target end-organ damage (echocardiography and electrocardiographic criteria of left ventricular hypertrophy, carotid intima-media thickness and proteinuria) and previous history of cerebrovascular events or peripheral vascular disease were recorded for all patients.

Repeat office blood pressure levels, repeat lipid levels, medication usage, antihypertensive treatment, statin therapy, and aspirin use were recorded throughout the trial, as were outcome measures of the composite endpoint that included all cardiovascular events and procedures plus development of renal impairment. Urine samples, collected in the morning after an overnight fast (10–12 h), were tested for blood, sugar, and protein, then divided into aliquots and stored at −20°C until analyzed.

The study was approved by the ethics committee at St. Mary's Hospital, London and Beaumont Hospital, Dublin (REC# 99/21), and adheres to the principles of the Declaration of Helsinki. All patients gave written informed consent.

In this proteomics study, we adopted a nested case-control design within the HACVD substudy of the ASCOT trial. The primary endpoint in this study was CAD, the definition of which included the following endpoints: fatal CAD, symptomatic nonfatal myocardial infarction and coronary revascularization. Controls for cases were individuals who had not experienced a cardiovascular disease endpoint during the study period (median observation time 5 years). All cases and controls were defined by presence of a urine sample from 1 to 1.5 years postrandomization, stored for biomarker assay. Eighty-seven percent of patients were white-European and to exclude any effects of ethnicity, selection of cases, and controls was restricted to this group. Subjects with established cardiovascular disease at baseline (including previous history of stroke, transient ischaemic attack, peripheral vascular disease) were excluded from this prospective study.

All remaining eligible cases (N = 37) from the HACVD cohort were included in this study, and matched (1:1) for age (± 2 years), sex, smoking, diabetes, and the same treatment arm of the ASCOT study, as described in Fig. 1.
2.2 Urinary proteomics

2.2.1 Sample handling

Samples were prepared as described previously 12. A 0.7 mL aliquot of urine was diluted with 0.7 mL of 2 mmol/L urea and 10 mmol/L NH₄OH containing 0.02% SDS. Proteins with a molecular mass >20 kDa were removed by filtering of samples using Centrisart ultracentrifugation filter devices (Sartorius, Gottingen, Germany) at 3000 × g until 1.1 mL filtrate was obtained. The filtrate was applied onto a PD-10 desalting column (Amersham Bioscience) equilibrated with 0.01% NH₄OH to remove urea, electrolytes and salts, and to enrich the polypeptides present. Before analysis all samples were lyophilized, stored at 4°C, and resuspended in HPLC-grade H₂O.

2.2.2 CE-MS studies

Figure 1 Hypertension associated cardiovascular disease (HACVD) urinary proteomic study population. *QC - quality control.
CE coupled to MS enables reproducible, robust, high resolution analysis of several thousand low molecular weight urinary proteins/peptides 21. CE-MS was performed as described previously 12 using a P/ACE MDQ CE system (Beckman Coulter) online coupled to a TOF mass spectrometer (microTOF; Bruker Daltonic). Data acquisition and MS acquisition were automatically controlled by the CE via contact close relays. Spectra were accumulated every 3 s over a range of mass-to-charge ratios from 350 to 3000.

2.2.3 Data processing

Data were analyzed using Mosaiques-Visu software; CE-MS peaks were detected using a S/N of at least four. The charge of each peak was calculated based on isotopic distributions and conjugated masses. Data were deconvoluted and mass spectral ion peaks from the same molecule at different charge states were combined and recorded as a single mass. MS data were normalized as described previously 22 to correct for technical variation such as signal suppression as well as biological variation such as differing urine concentrations due to hydration status of the patient at time of collection. In particular, reference signals of >1700 urinary peptides were used for CE time calibration and MS signal intensities (ion counts) were normalized relative to 29 “housekeeping” peptides with small RSD. For TOF MS mass calibration, 80 reference masses exactly determined by Fourier transform ion cyclotron resonance MS were used. The resulting peak list characterizes each peptide by its molecular mass (Da) and CE migration time, normalized signal intensities are used as measure for relative abundance. Data were entered into a Microsoft SQL database for comparison with other samples and for further analysis. MS peaks from different samples were presumed identical if mass deviation was ≤50 ppm for small or ≤75 ppm for larger peptides, and the migration time deviation was 2 min.

2.2.4 Data analysis

Our groups have previously developed a 238-peptide panel (CAD238) using CE-MS that is associated with CAD in a cross-sectional study 12. This panel was assessed for its predictive value in this study. A numerical value, known as a “classification factor,” was generated using Mosaiques Visu software for more straightforward statistical analysis of the resulting polypeptide picture for each individual subject; lower classification factor values considered to be “healthier.” Cases and controls were compared using paired t-test and linear regression analyses. We then performed survival analysis to determine whether classification factors from the urinary proteomic panel could be predictive of
cardiovascular events using the CAD$_{238}$ model. Classification factors were split into values greater than the mean, and less than the mean for each model. We also performed survival analysis, in the same way, for classification factors from other previously derived models for CKD (CKD$_{273}$ 23), heart failure 16, and stroke 15 as negative controls. We were not expecting these panels to be of use in discriminating between cases and controls for coronary artery disease.

2.2.5 Statistical analysis

Analysis of clinical parameters and the classification factors derived from urinary proteome analysis were performed using SPSS software, (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp). Parametric and nonparametric tests for comparison between groups were applied as appropriate. Data are given as mean ± SD, median (interquartile range) or percentage as appropriate. Comparisons were made using paired t-test, McNemar's test and linear regression as appropriate. Kaplan–Meier survival analysis was performed to compare classification factors and likelihood of experiencing a cardiovascular event. The nonparametric Mantel Cox log rank test was used to compare survival distributions between groups according to CAD$_{238}$ classifier (</>mean, as described below). Cox proportional hazards analysis was used to analyze the effect of CAD classifier along with other covariates on survival. p-values <0.05 were considered significant and AUC cut off >0.6 was used.

3 Results

Of the 37 cases and 37 matched controls included in this study, sixty urine samples passed quality control for proteomic analysis (Table 1, Fig. 1). These 60 samples comprised 32 cases who experienced CAD events and 28 controls, with 88% male and a mean age 64 ± 5 years (Table 1). There were no significant differences in mean age, gender, blood pressure, cholesterol, smoking history, or drug treatment arms between cases and controls.
### Table 1. Baseline characteristics of cases and controls passing quality control

<table>
<thead>
<tr>
<th></th>
<th>Controls n = 28</th>
<th>95% CI</th>
<th>Cases n = 32</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yrs)</td>
<td>64 ± 5</td>
<td>(62, 66)</td>
<td>63 ± 6</td>
<td>(61, 65)</td>
<td>0.623</td>
</tr>
<tr>
<td>Sex male</td>
<td>24 (86%)</td>
<td></td>
<td>29 (91%)</td>
<td></td>
<td>0.625</td>
</tr>
<tr>
<td>Time in study (months)</td>
<td>67 ± 7</td>
<td>(64, 70)</td>
<td>34 ± 16</td>
<td>(28, 40)</td>
<td>0.000</td>
</tr>
<tr>
<td>SBPa (mmHg)</td>
<td>161 ± 20</td>
<td>(154, 169)</td>
<td>162 ± 17</td>
<td>(156, 168)</td>
<td>0.874</td>
</tr>
<tr>
<td>DBPb (mmHg)</td>
<td>92 ± 9</td>
<td>(89, 95)</td>
<td>93 ± 11</td>
<td>(89, 97)</td>
<td>0.588</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>5.7 ± 0.9</td>
<td>(5.3, 6.0)</td>
<td>5.6 ± 1.0</td>
<td>(5.3, 6.0)</td>
<td>0.958</td>
</tr>
<tr>
<td>HDLc (mmol/L)</td>
<td>1.3 ± 0.4</td>
<td>(1.1, 1.4)</td>
<td>1.3 ± 0.4</td>
<td>(1.1, 1.4)</td>
<td>0.977</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>95 ± 13</td>
<td>(90, 100)</td>
<td>104 ± 20</td>
<td>(97, 111)</td>
<td>0.066</td>
</tr>
<tr>
<td>Smoker</td>
<td>7 (25%)</td>
<td></td>
<td>6 (19%)</td>
<td></td>
<td>0.508</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>17 (61%)</td>
<td></td>
<td>16 (50%)</td>
<td></td>
<td>0.607</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>11 (39%)</td>
<td></td>
<td>11 (34%)</td>
<td></td>
<td>0.581</td>
</tr>
</tbody>
</table>

*a* SBP, systolic blood pressure. *b* DBP, diastolic blood pressure. *c* HDL, high-density lipoprotein.

The CAD$_{238}$ classifier appeared to be different between cases with CAD events and controls at baseline (–0.432 ± 0.326 versus –0.587 ± 0.297) although the difference was not statistically significant (p = 0.170).

We then performed survival analysis to assess the model in its ability to detect cases from controls at a given classifier level cut-off. We used mean classifier value to divide subjects into two groups; those with a classifier value of greater
than the mean were compared with those with classifier values below the mean. The Mantel Cox test revealed a significant difference between the hazard functions of the two groups (test statistic 5.35, p-value 0.021) for the CAD$_{238}$ model (Fig. 2). Cox proportional hazards model with age and gender as covariates revealed that the hazard for CAD score >mean was 2.38 times that of those <mean, p-value 0.017. As expected, on further survival analysis of matched (age, gender, treatment arm, diabetes, smoking status) and unmatched (blood pressure, cholesterol level) variables, no other variable showed significant differences between groups with versus without CAD events (data not shown).

**Figure 2** Kaplan–Meier curve for the CAD$_{238}$ model. Subjects were classified as < or > the mean value of the CAD$_{238}$ classification factor. Log rank (Mantel Cox) test statistic 5.35, p = 0.021.

In order to confirm the predictive value of the CAD$_{238}$ panel for the specific outcomes in this study we also tested proteomic panels related to other conditions. As expected, the panels that predict CKD, heart failure, and stroke were neither different at baseline (cases versus controls: −0.069 ± 0.388 versus −0.095 ± 0.236, p = 0.927; 0.282 ± 0.663 versus 0.114 ± 0.569, p = 0.333; and −0.114 ± 0.679 versus −0.140 ± 0.528, p = 0.873, respectively) (Supporting Information Fig. 1) nor on survival analysis (log rank Mantel-Cox p = 0.690, p =
0.386, and $p = 0.874$, respectively), for the outcome “CAD event” (Supporting Information Fig. 2).

We subsequently unblinded the cohort and studied all urinary polypeptides identified on CE-MS analysis individually. We found 190 individual peptides out of 1501 urinary peptides that were different between cases and controls at baseline at the AUC >0.6 cut-off (Supporting Information Table 1). Many of these peptides have previously been associated with cardiovascular disease, most notably fragments of collagens.

Twenty-five of these 190 peptides were also components of the 238 peptides comprising the CAD$_{238}$ panel (Table 2), including fragments of membrane associated progesterone receptor component 1 (PGRC1), prostaglandin-H2 D-isomerase (PTGDS), collagen alpha-1(I) chain (CO1A1), collagen alpha-1(III) chain (CO3A1), collagen alpha-1 (XVI) chain (COGA1), plexin domain-containing protein 2 (PXDC2), beta 1,3-galactosyltransferase 6 (B3GT6), and retinol-binding protein 4 (RBP4).

**Table 2.** The 25 urinary polypeptide markers showing significant differences between cases and controls in this study, which were part of the previously derived CAD$_{238}$ panel

<table>
<thead>
<tr>
<th>Protein name</th>
<th>SwissProt/ TrEMBLE name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular matrix</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1(II) chain</td>
<td>CO2A1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1(III) chain</td>
<td>CO3A1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1(V) chain</td>
<td>CO5A1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1 (XVII) chain</td>
<td>COHA1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1 (XVI) chain</td>
<td>COGA1_HUMAN</td>
</tr>
<tr>
<td>Protein name</td>
<td>SwissProt/ TrEMBLE name</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Collagen alpha-2(I) chain</td>
<td>CO1A2_HUMAN</td>
</tr>
<tr>
<td>Haemostasis and erythropoiesis</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>FIBB_HUMAN</td>
</tr>
<tr>
<td>Hemoglobin subunit beta</td>
<td>HBB_HUMAN</td>
</tr>
<tr>
<td>Hemoglobin subunit delta</td>
<td>HBD_HUMAN</td>
</tr>
<tr>
<td>Inflammation and immune response</td>
<td></td>
</tr>
<tr>
<td>Beta-2-microglobulin</td>
<td>B2MG_HUMAN</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC_HUMAN</td>
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<tr>
<td>Ig gamma-1 chain C region</td>
<td>IGHG1_HUMAN</td>
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<tr>
<td>Ig lambda-2 chain C regions</td>
<td>LAC2_HUMAN</td>
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<tr>
<td>Signaling</td>
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<tr>
<td>Membrane associated progesterone receptor component 1</td>
<td>PGRC1_HUMAN</td>
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<tr>
<td>Prostaglandin-H2 D-isomerase</td>
<td>PTGDS_HUMAN</td>
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<tr>
<td>Zinc finger protein 653</td>
<td>ZN653_HUMAN</td>
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<tr>
<td>Other</td>
<td></td>
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<tr>
<td>Beta 1,3-galactosyltransferase 6</td>
<td>B3GT6_HUMAN</td>
</tr>
<tr>
<td>Neurosecretory protein VGF</td>
<td>VGF_HUMAN</td>
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</tbody>
</table>
Protein name | SwissProt/ TrEMBLE name
--- | ---
Plexin domain-containing protein 2 | PXDC2_HUMAN
Polymeric-immunoglobulin receptor | PIGR_HUMAN
Retinol-binding protein 4 | RET4_HUMAN
Sodium/potassium-transporting ATPase subunit gamma | ATNG_HUMAN
Uromodulin | UROM_HUMAN
Zinc-alpha-2-glycoprotein | ZA2G_HUMAN

4 Discussion

We have already used CE-MS-based urinary proteomics in our clinical studies in a wide range of conditions 15, 16, 23, 24. The relatively high throughput with this technique, the stability of the urinary proteome and the noninvasiveness of urine sampling are some of the key advantages of this approach. Given the large number of biomarkers that can be assessed with proteomics it is not unreasonable to propose that they provide information on different pathophysiological aspects of the conditions including earlier and later steps in disease development. We have therefore proposed that proteomic biomarkers can be used both to detect early stages in the disease process and to predict development of more advanced stages including clinically overt disease in asymptomatic patients.

The most robust data to support this hypothesis derives from our work in chronic kidney disease where a panel of 273 urinary biomarkers (CKD273) has been found to differentiate between patients with normal and impaired renal function 25. The same panel has been found to predict the development of chronic kidney disease. In particular Roscioni et al. 26 have shown that it predicts the development of diabetic nephropathy in patients with type 2 diabetes earlier and with greater sensitivity and specificity than currently used predictors, including microalbuminuria. Similarly, we have recently shown that a proteomic signature that was developed in patients with diastolic dysfunction
but without symptoms of heart failure also differentiates between patients with overt heart failure and healthy controls \textsuperscript{16}, demonstrating that pathophysiological processes that provide a link between early and advanced disease processes can be represented in the urinary proteomic signatures. We have excluded people with a history of stroke and have therefore not looked at stroke events. The stroke panel itself was originally derived to differentiate acute stroke from stroke-like conditions. We used it here as a negative control. We also used the heart failure and CKD panels used in this manner as we were similarly not expecting positive findings for these panels in our study. Reassuringly these panels were negative in predicting between cases and controls for coronary artery disease.

In the present study, we have taken our clinical proteomic studies into CAD one step further, from a cross-sectional study to prediction by evaluating the potential of a panel of urinary polypeptides to predict future coronary events. This panel (CAD\textsubscript{238}) was developed and extensively validated in cross-sectional studies of patients with CAD versus healthy controls. In order to provide the best balance between sensitivity and specificity the model was built on data from patients with a wide range of disease severity \textsuperscript{12}, covering therefore early and more advanced disease processes. The main result of the present study is that in patients who do not have overt cardiovascular disease at baseline but are at risk to develop such disease and associated events due to their risk profile, the CAD\textsubscript{238} panel differentiates between patients who had CAD events within the study period and those who did not. The nested case-control design of our study allowed for rigorous matching for cardiovascular risk factors and treatment arm and thereby considerably reduced confounding. We used the ASCOT study specifically because one might reasonably have predicted cardiovascular events to occur in this cohort with higher-than-average prevalence of CAD risk factors \textsuperscript{18}.

We have also demonstrated that not all of the 238 markers in the original CAD\textsubscript{238} panel are significantly associated with CAD events. Looking at all urinary polypeptides, 25 of the 190 markers that were differentially expressed between cases and controls were also part of the CAD\textsubscript{238} panel. We are not surprised about this seemingly small overlap. The CAD\textsubscript{238} panel was developed to represent different stages of CAD severity including triple vessel CAD requiring CABG surgery that was not the case in the patients selected for the present study. It appears therefore plausible that only some of the markers in the CAD\textsubscript{238} panel were found to be different between cases and controls in our
present study, and it can be hypothesized that these markers reflect earlier stages of CAD that had the potential to progress to clinically overt disease during the course of the follow-up period.

It is also important to highlight the fact that these proteins are derived not only from the kidney, but potentially from all organs. In this way the urine can depict systemic processes such as turnover in the extracellular matrix and the pattern may change over time and depending on the disease 14. The urinary biomarkers identified relate to key molecular components of CAD. Indeed collagen types 1 and 3 have previously been found in urinary proteomic studies relating to cardiovascular disease 12. This study found decreased urinary excretion of certain collagen type 1 and 3 alpha chain fragments but increased excretion of specific collagen type 1 and 3 alpha chain fragments with a C-terminal GxPGP motif. Another example is retinol-binding protein 4 (RBP4) which has been associated with metabolic syndrome in both sexes, prior cerebrovascular disease in men, and it is also thought that circulating RBP4 could be a marker of metabolic complications, atherosclerosis and cardiovascular disease 27, 28. RBP4 is an adipokine, and there is emerging evidence that adipokines are involved in the development of cardiovascular disease and provide a link between insulin resistance, obesity, and inflammation 29. The adipokine zinc-alpha-2-glycoprotein (ZA2G) was also identified among the 25 urinary polypeptide markers showing significant difference between cases and controls. We also found differences in expression of Fibrinogen beta chain in keeping with beta-chain synthesis being a key step in the production of mature fibrinogen 30. Fibrinogen is known to play an important role in inflammation, atherogenesis, and thrombogenesis. Several polymorphisms have been identified in the genes encoding the different fibrinogen chains that determine plasma levels of fibrinogen, however, plasma fibrinogen level, and cardiovascular risk may be more dependent on interactions with environment and genetics (e.g. moderate alcohol intake may lower plasma fibrinogen concentration) 30.

Even in light of the pathophysiological significance of individual protein fragments we would like to reinforce the message that for immediate clinical diagnostic and prognostic purposes the global view on a panel of proteomic markers may be of greater importance than individual markers, although their pathophysiological relevance is reassuring. Another stream of information may derive from the proteases that are responsible for the generation of specific urinary peptides from their source proteins. Protease activity prediction has been proposed previously and appropriate tools have been developed 31. It
would be beyond the scope of this study to predict and validate protease activity but we would like to reinforce the message that the urinary proteome provides information beyond individual peptides, peptide patterns, and source proteins.

The principal limitation of the study is that of a relatively small sample in a nested case-control design. Clearly a larger study will need to be performed to get a better impression of the potential predictive value in a clinical setting. We also acknowledge that our data have not been replicated in an independent cohort. On the other hand, we have applied extremely strict criteria for the selection and matching of cases and controls and performed the CE-MS analysis in a blinded fashion. We used samples from a well-controlled clinical trial with validated endpoints adding further strength to the design.

In summary, individualized cardiovascular risk prediction remains an unmet clinical need in patients with hypertension and other risk factors. In a substudy of the ASCOT study we have shown that a panel of urinary polypeptides that was originally developed to differentiate between patients with and without CAD can also be predictive of coronary events in asymptomatic subjects with hypertension. Studying multiple biomarkers at a time, proteomics has the potential to detect subclinical disease and thereby improve risk stratification and targeted treatment.

A number of questions remain to be answered to clarify the clinical utility of this proteomic panel. These include how best to identify patients who would benefit from proteomic testing, the positive and negative predictive values of the test, and the comparison of proteomics with other emerging biomarkers of cardiovascular risk.

**Acknowledgments**

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*The authors have declared the following potential conflict of interest: H.M. is founder and co-owner of Mosaiques Diagnostics, which developed the CE-MS technology and the Mosaiques-Visu software.*
5 References


