URINARY PROTEOMIC BIOMARKERS TO PREDICT CARDIOVASCULAR EVENTS

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Abbreviations:
ASCOT Anglo-Scandinavian Cardiac Outcomes Trial
CAD coronary artery disease,
CKD chronic kidney disease
MI myocardial infarction

Key words: biomarker; cardiovascular risk; urinary proteomics

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Potential clinical significance of the study in terms of clinical need, goals and how the study moves the field forward

Individualised 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.

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) sub-study population of the ASCOT 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 analysed using capillary electrophoresis (CE) coupled to Micro-TOF mass spectrometry (MS). A previously developed 238-marker CE-MS model for diagnosis of CAD (CAD238) was assessed for its predictive potential.

Results: Sixty urine samples (32 cases; 28 controls; 88% male, mean age 64±5 years) were analysed. There was a trend towards healthier values in controls for the CAD model classifier (-0.432±0.326 vs -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 CAD238.
Conclusion & 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.

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.

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

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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 hypothesised 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).

Methods

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 & 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 non-cardiovascular 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 non-fasting cholesterol level of ≤ 6.5 mmol/L were recruited into ASCOT-LLA, and randomized to either atorvastatin 10 mg or placebo (19).

A nested case-control design was used. The population examined in this study was the Hypertension Associated Cardiovascular Disease (HACVD) cohort; an intensively phenotyped sub-study of (ASCOT) (20). The HACVD sub-study involved intensive phenotyping of 1,395 subjects, along with DNA collection, with the intention of identifying unrecognised genetic influences on the development of hypertension associated cardiovascular disease (20). 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 which included all cardiovascular events and procedures plus development of renal impairment. Urine samples, collected in the morning after an overnight fast (10 to 12 hours), were tested for blood, sugar and protein, then divided into aliquots and stored at -20°C until analysed.
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 non-fatal myocardial infarction (MI) and coronary revascularisation. 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 1-1.5 years post-randomisation, stored for biomarker assay. 87% 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 Figure 1.

**Urinary Proteomics**

**Sample Handling**

Samples were prepared as described previously (12). A 0.7mL aliquot of urine was diluted with 0.7mL of 2 mmol/L urea and 10 mmol/L NH₄OH containing 0.02% SDS. Proteins with a molecular mass >20kDa were removed by filtering of samples using Centrisart ultracentrifugation filter devices (Sartorius, Gottingen, Germany) at 3000g until 1.1mL 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 high-performance liquid chromatography-grade H₂O.
CE-MS Studies

Capillary-electrophoresis (CE) coupled to mass spectrometry (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 capillary electrophoresis system (Beckman Coulter) online coupled to a time-of-flight 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 seconds over a range of mass-to-charge ratios from 350 to 3000.

Data processing

Data were analysed using Mosaiques-Visu software; CE-MS peaks were detected using a signal-to-noise ratio of at least 4. 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 relative standard deviation. For time-of-flight MS mass calibration, 80 reference masses exactly determined by Fourier transform ion cyclotron resonance mass spectrometry 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 minutes.
Data analysis

Our group have previously developed a 238-peptide panel (CAD\textsubscript{238}) using CE-MS which is associated with CAD in a cross-sectional study (12). This panel was assessed for its predictive value in this study. For comparison, three other models, devised in a similar manner (for chronic kidney disease (CKD\textsubscript{273}) (23), heart failure (16) and stroke (15)) were also assessed in this population for their predictive values. 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\textsubscript{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, heart failure and stroke as negative controls. We were not expecting these panels to be of use in discriminating between cases and controls for coronary artery disease.

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 non-parametric 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 non-parametric Mantel Cox logrank test was used to compare survival distributions between groups according to CAD\textsubscript{238} classifier (</>/mean, as described below). Cox proportional hazards analysis was used to analyse 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.
Results

Of the 37 cases and 37 matched controls included in this study, sixty urine samples passed quality control for proteomic analysis (Table 1, Figure 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.

The CAD238 classifier appeared to be different between cases with CAD events and controls at baseline (-0.432±0.326 vs -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 CAD238 model (Figure 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 vs without CAD events (data not shown).

In order to confirm the predictive value of the CAD238 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 vs controls: -0.069±0.388 vs -0.095±0.236, P=0.927; 0.282±0.663 vs 0.114±0.569, P=0.333; and -0.114±0.679 vs -0.140±0.528, P=0.873, respectively) (Supplementary Figure S1) nor on survival analysis (log rank Mantel-Cox P=0.690, P=0.386, and P=0.874, respectively), for the outcome "CAD event" (Supplementary Figure S2).
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 (Supplementary Table S1). 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 CAD238 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).

**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 non-invasiveness 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 (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 (CAD238) was developed and extensively validated in cross-sectional studies of patients with CAD vs 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 (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. By matching on such major risk factors, the potential for confounding was considerably reduced. 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 (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 which 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 hypothesised 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 they are involved in the development of cardiovascular disease. This is not surprising as they are already known to 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. Fibrinogen beta chain was also found to be different which is unsurprising considering that beta-chain synthesis is 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 fibrinogen chain genes 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 that was designed to take a cross-sectional proteomic classifier to longitudinal prediction 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.

**Concluding remarks**

Individualised cardiovascular risk prediction remains an unmet clinical need in patients with hypertension and other risk factors. In a sub-study 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. Nevertheless, we have here presented an important second step in the development of a potentially clinically useful biomarker by taking it from the cross-sectional to the longitudinal level.

**Acknowledgements**

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**Funding**

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**Conflicts of interest**

H.M. is founder and co-owner of Mosaiques Diagnostics, which developed the CE-MS technology and the Mosaiques-Visu software.

**Reference List**


(5) Sehestedt T, Jeppesen J, Hansen TW, Wachtell K et al., Risk prediction is improved by adding markers of subclinical organ damage to SCORE. *Eur Heart J* 2010; 31(7):883-891.


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**Figure 1.** Hypertension Associated Cardiovascular Disease (HACVD) Urinary Proteomic Study population.

*QC = quality control

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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.
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>
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<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>29 (91%)</td>
<td></td>
<td></td>
<td>0.625</td>
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<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>SBP † (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>DBP ‡ (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>HDL § (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>6 (19%)</td>
<td></td>
<td></td>
<td>0.508</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>17 (61%)</td>
<td>16 (50%)</td>
<td></td>
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<td>0.607</td>
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<tr>
<td>Atorvastatin</td>
<td>11 (39%)</td>
<td>11 (34%)</td>
<td></td>
<td></td>
<td>0.581</td>
</tr>
</tbody>
</table>

†SBP – systolic blood pressure, ‡DBP – diastolic blood pressure, §HDL – high-density lipoprotein
Table 2. The 25 urinary polypeptide markers showing significant differences between cases and controls in this study, which were part of the previously derived CAD238 panel

<table>
<thead>
<tr>
<th>PROTEIN NAME</th>
<th>Swiss Prot/TrEMBLE name</th>
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</thead>
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<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>Collagen alpha-2(I) chain</td>
<td>CO1A2_HUMAN</td>
</tr>
<tr>
<td><strong>Haemostasis and erythropoiesis</strong></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><strong>Inflammation and immune response</strong></td>
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<tr>
<td>Beta-2-microglobulin</td>
<td>B2MG_HUMAN</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC_HUMAN</td>
</tr>
<tr>
<td>Ig gamma-1 chain C region</td>
<td>IGHG1_HUMAN</td>
</tr>
<tr>
<td>Ig lambda-2 chain C regions</td>
<td>LAC2_HUMAN</td>
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<td><strong>Signalling</strong></td>
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<td>Prostaglandin-H2 D- isomerase</td>
<td>PTGDS_HUMAN</td>
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<td>Zinc finger protein 653</td>
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### Other

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<td>B3GT6_HUMAN</td>
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<td>Neurosecretory protein VGF</td>
<td>VGF_HUMAN</td>
</tr>
<tr>
<td>Plexin domain-containing protein 2</td>
<td>PXDC2_HUMAN</td>
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<tr>
<td>Polymeric-immunoglobulin receptor</td>
<td>PIGR_HUMAN</td>
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<td>Retinol-binding protein 4</td>
<td>RET4_HUMAN</td>
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<tr>
<td>Sodium/potassium - transporting ATPase subunit gamma</td>
<td>ATNG_HUMAN</td>
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<tr>
<td>Uromodulin</td>
<td>UROM_HUMAN</td>
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<tr>
<td>Zinc-alpha-2-glycoprotein</td>
<td>ZA2G_HUMAN</td>
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