

# Urinary Proteomic Biomarkers in Coronary Artery Disease\*

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Urinary proteomics is emerging as a powerful non-invasive tool for diagnosis and monitoring of variety of human diseases. We tested whether signatures of urinary polypeptides can contribute to the existing biomarkers for coronary artery disease (CAD). We examined a total of 359 urine samples from 88 patients with severe CAD and 282 controls. Spot urine was analyzed using capillary electrophoresis on-line coupled to ESI-TOF-MS enabling characterization of more than 1000 polypeptides per sample. In a first step a “training set” for biomarker definition was created. Multiple biomarker patterns clearly distinguished healthy controls from CAD patients, and we extracted 15 peptides that define a characteristic CAD signature panel. In a second step, the ability of the CAD-specific panel to predict the presence of CAD was evaluated in a blinded study using a “test set.” The signature panel showed sensitivity of 98% (95% confidence interval, 88.7–99.6) and 83% specificity (95% confidence interval, 51.6–97.4). Furthermore the peptide pattern significantly changed toward the healthy signature correlating with the level of physical activity after therapeutic intervention. Our results show that urinary proteomics can identify CAD patients with high confidence and might also play a role in monitoring the effects of therapeutic interventions. The workflow is amenable to clinical routine testing suggesting that non-invasive proteomics analysis can become a valuable addition to other biomarkers used in cardiovascular risk assessment. *Molecular & Cellular Proteomics* 7:290–298, 2008.

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Coronary artery disease (CAD)<sup>1</sup> is a leading cause of morbidity and mortality worldwide. The underlying molecular causes are still largely unknown but are likely to involve alterations in gene and protein expression (1). Despite multiple clinical, electrographic, and biochemical characteristics, there are subgroups of patients who progress to severe, life-threatening CAD without many symptoms and signs (2). For example, patients with type 2 diabetes and the elderly frequently suffer from silent myocardial infarctions with significantly increased risk of complications (3). Early diagnosis of CAD in its presymptomatic stage would allow for better, targeted, and hence more effective primary prevention as compared with current clinical recommendations. Proteomics is increasingly used to examine dynamic changes in protein expression providing new insights into cellular processes. Moreover proteomics analyses have already resulted in the identification of clinically useful biomarkers and can assist in diagnosis and disease staging (1, 4, 5). Substances contained in body fluids hold an abundance of information and can be used as a dynamic and concurrent gauge for monitoring the well-being of an organism. Urine presents a rich source of information related to the functioning of many internal organs (5–7), and the appearance of certain proteins in the blood stream may result in their appearance in the urine either in the intact form or as peptide fragments. The protein and peptide composition of the urine is determined by the function of the glomerular filtration apparatus, proximal tubular absorption of ultrafiltered proteins, and the capacity of the brush border and lysosomal proteolytic machinery to degrade filtered proteins (8). Therefore, detection of one or several proteins or polypeptides may provide a signature of a particular pathological process (7).

We hypothesized that proteomics analysis of urine should yield a panel of biomarker peptides useful as additional tools for the diagnosis and monitoring of CAD. Furthermore we aimed to obtain sequences of biomarkers of the CAD signature panel to gain insight into pathogenetic mechanisms and facilitate comparison with currently used biochemical mark-

<sup>1</sup> The abbreviations used are: CAD, coronary artery disease; CE, capillary electrophoresis; ETD, electron transfer dissociation; ROC, receiver operating characteristic; AUC, area under the ROC curve; CI, confidence interval; DTA, .dta file; LDL, low density lipoprotein; HDL, high density lipoprotein.

ers. Capillary electrophoresis on-line coupled to electrospray ionization-time-of-flight mass spectrometry (CE-ESI-TOF MS) seems ideally suited for this purpose because of its non-invasive nature, high resolution, and amenability for future adaptation to clinical laboratory analysis (5).

#### EXPERIMENTAL PROCEDURES

**Subjects and Procedures**—We enrolled 88 patients with CAD confirmed by coronary angiography. Patients were recruited at the Western Infirmary, Glasgow, UK. Eighty-two of the 88 patients were reassessed after a further 14 weeks, one patient died, and five patients refused to participate at a follow-up examination. At both assessments blood and midstream spot urine samples were collected. Thirty-two subjects with no history of angina, CAD, or peripheral artery disease who were recruited from a local health club and from surgical wards at Gartnavel General Hospital, Glasgow, UK served as controls. Plasma total cholesterol, low density lipoproteins, high density lipoproteins, triglycerides, high sensitivity C-reactive protein, and serum creatinine were assessed using standard biochemical methods. The modification of diet in renal disease formula was used for the estimation of glomerular filtration rate in study participants (9).

Vascular stiffness was assessed by two methods. First, pulse contour analysis of the diastolic pressure decay was used to estimate large (C1) and small artery compliance (C2; HDI/Pulse Wave CR2000, HDI Inc., Eagan, MN) based on a three-element Windkessel model (10). Second, the augmentation index of the central aorta was derived from the radial pulse waveform using a generalized transfer function (SphygmoCor pulse wave analysis system, AtCor Medical, West Ryde, New South Wales, Australia) (11). The augmentation index was calculated from the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak.

The study was approved by the West Glasgow Ethics Committee, and all subjects gave written informed consent. This study was designed according to current guidelines for studies on clinical proteomics (12) and the minimum information about proteomics experiments (MIAPE) (13).

To exclude the effect of medication, 17 paired urine samples from age- and sex-matched patients with hypertension and type 2 diabetes, but without albuminuria, before and 12 weeks after commencing treatment with the angiotensin-converting enzyme inhibitor ramipril (5–10 mg once daily) were evaluated. To rule out center specific bias, samples from 233 new appointees at the University of Hannover who were free of self-reported illness were also analyzed. Detailed characteristics of all patients and controls are shown in Table I with additional data on 233 healthy university recruits and 18 ramipril patients shown in Table II.

**Physical Activity Levels in CAD Patients**—At follow-up examination, self-reported physical activity was assessed. The physical activity was graded into two categories: *no* regular physical activity (patients mainly confined indoors) or *low grade* physical activities like walking on flat terrain or non-strenuous gardening and a *very active group* with hiking, biking, and golfing several times a week. This classification was independently validated by physiotherapists who categorized patients' activity levels according to clinical data ( $r = 0.379$ ,  $p = 0.006$ ). Furthermore physiotherapists performed an incremental shuttle walk test (14) after base-line examination in 52 of the 88 patients. There was a significant correlation between metabolic equivalent obtained by the test and self-reported activity ( $r = 0.399$ ,  $p = 0.003$ ).

**Urine Sample Preparation and CE-MS Analysis**—After collection, all the spot urine samples were frozen at  $-80^{\circ}\text{C}$  until analysis. For proteomics analysis samples were prepared as described previously (15, 16). CE-MS analysis was performed as described previously (15–17) using a P/ACE MDQ capillary electrophoresis system (Beck-

man Coulter, Fullerton, CA) on-line coupled to a Micro-TOF MS instrument (Bruker Daltonics, Bremen, Germany). The performance of the sample preparation procedure as well as the analytical performance of the instrumental setup was evaluated (5, 18). The average recovery of the sample preparation procedure is  $\sim 85\%$  with a detection limit of  $\sim 1$  fmol. The monoisotopic mass signals could be resolved for  $z \leq 6$ . The mass accuracy of the CE-TOF-MS method was determined to be  $< 25$  ppm for monoisotopic resolution and  $< 100$  ppm for unresolved peaks ( $z > 6$ ). The precision of the analytical method was determined by assessing (a) the reproducibility achieved for repeated measurement of the same aliquot and (b) the reproducibility achieved for repeated preparation and measurement of the same urine sample (5, 18). The 200 most abundant polypeptides were detected with a rate of 98%. The performance of the analytical system over time was assessed with consecutive measurements of the same aliquot over a period of 24 h. No significant loss of polypeptides was observed implying the stability of the CE-MS setup, the postpreparative stability of the urine samples at  $4^{\circ}\text{C}$ , and their resistance to e.g. oxidizing processes or precipitation (5, 18).

**Data Processing and Cluster Analysis**—Data processing and cluster analysis were performed as described previously (15, 19). Only signals observed in a minimum of three consecutive spectra with a minimum signal-to-noise ratio of 4 were considered. Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using either the distance between resolved isotope peaks of the ion or according to conjugated signals for unresolved isotope peaks (MosaïquesVisu software (16, 18)). In addition, migration time and ion signal intensity (amplitude) were normalized using internal polypeptide standards (15). The resulting peak list characterizes each polypeptide by its molecular mass (kDa), normalized migration time (min), and normalized signal intensity. All detected polypeptides were deposited, matched, and annotated in a Microsoft SQL (structured query language) database, allowing further analysis and comparison of multiple samples (patient groups). Polypeptides within different samples were considered identical if the mass deviation was less than 100 ppm and the migration time deviation was less than 3%. CE-MS data of all individual samples can be accessed in the supplemental table.

**Definition of Biomarkers and Sample Classification**—For biomarker panel definition, we used polypeptides that were found in more than 75% of the urine samples in at least one of the different groups of the training set (e.g. CAD or healthy controls). Polypeptides fulfilling this criterion were further evaluated using receiver operating characteristic (ROC) statistics (20). The amplitude distribution of the CE-MS data of polypeptides present in the samples was used as the ROC variable, and the affiliation to a diagnostic group (i.e. CAD or healthy control) was used as the classification variable. The obtained area under the ROC curve (AUC) value of the analysis of a given polypeptide was interpreted as a measure of its discriminatory potential. An initial list of potential marker candidates was further refined using the Mann-Whitney test with  $p \leq 0.05$  as the significance level. Model establishment and sample classification were performed by using a linear classifier algorithm according to  $F = \sum_i c_i \log A_i$  with  $F$  as classification factor,  $c_i$  as classification coefficient, and  $A_i$  as amplitude of the CE-MS signal of the marker  $i$ . The algorithm generates a classification model based on polypeptides that are best suited to discriminate between two defined sample groups. Models consist of fewer biomarkers than samples to avoid overfitting of models. The probability to have CAD at a given classification factor  $F$  taking into account the related probability for a negative diagnosis was calculated according to Equation 1.

$$P_{\text{CAD}} = \frac{1}{1+X} \text{ with } X = \frac{\text{S.D.}_{\text{CAD}}}{\text{S.D.}_{\text{HC}}} e^{\frac{(F_{\text{CAD}}^{\text{mean}} - F)^2}{2\text{S.D.}_{\text{CAD}}^2} - \frac{(F_{\text{HC}}^{\text{mean}} - F)^2}{2\text{S.D.}_{\text{HC}}^2}} \quad (\text{Eq. 1})$$

**Pattern Composition**—For the first phase of the study a training set was established. The training set consisted of 50 urine samples from randomly selected subjects, 30 CAD patients, and 20 control subjects, respectively. The first step of biomarker selection led to a set of 187 potential CAD-specific polypeptides.

In a second step, these preselected polypeptides were compared with 233 urine samples from healthy volunteers from different centers to eliminate polypeptides that may show center-specific bias. To exclude the effect of medication on constituting markers, an additional control group of patients before ( $n = 15$ ) and after ( $n = 17$ ) 12-week treatment with the angiotensin-converting enzyme ramipril was used to refine the selected polypeptides. Polypeptides that showed up/down-regulation of CE-MS signal intensity in direct comparison of both groups and in addition a uniform behavior in pairwise comparison in the majority of patients were considered as medication artifacts and eliminated.

Subsequently the established pattern of 15 polypeptides was evaluated in a blinded assessment of 59 urine samples: 47 samples from patients with CAD and 12 samples from healthy controls. All samples were examined using the CAD panel. Seventy-six urine samples from follow-up examination were also evaluated using the CAD panel.

**Sequencing of Polypeptides**—Peptide sequencing was performed using an LTQ-Orbitrap™ hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Dionex Ultimate 3000 nanoflow system and a nano-electrospray ion source. Peptide separation took place on a 5- $\mu\text{m}$   $\text{C}_{18}$  nanocolumn (NanoSeparations, Nieuwkoop, Netherlands) in a precolumn setup using a flow rate of 5  $\mu\text{l}/\text{min}$  followed by a flow of 250  $\text{nl}/\text{min}$  and a linear gradient (60 min) from 2 to 50% MeCN in  $\text{H}_2\text{O}$  (0.1% formic acid). The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from  $m/z$  300 to 2000) were acquired in the Orbitrap with resolution  $R = 60,000$  at  $m/z$  400 (target value of 500,000 charges in the linear ion trap). The most intense ions were sequentially isolated for fragmentation in the linear ion trap using collisionally induced dissociation and the detection took place either in the linear ion trap (parallel mode; target value 10,000) or in the Orbitrap (target value of 500,000). Orbitrap MS/MS were acquired with resolution  $R = 15,000$  at  $m/z$  400. General mass spectrometric conditions were: electrospray voltage, 1.6 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 225 °C; collision gas pressure, 1.3 millitorrs; normalized collision energy, 32% for MS/MS. The ion selection threshold was 500 counts for MS/MS.

Further analysis was performed using instruments with electron transfer dissociation (ETD) capability (21–23). Upon arrival, samples were resuspended (50  $\mu\text{l}$  of 100 mM acetic acid) and bomb-loaded onto a  $360 \times 75\text{-}\mu\text{m}$  microcapillary precolumn that was connected to a  $360 \times 50\text{-}\mu\text{m}$  analytical column with a  $\sim 1\text{-}\mu\text{m}$  tip pulled with a laser puller (both columns were packed in-house with  $\sim 5\text{--}8$  cm of  $\text{C}_{18}$  resin). Peptides were separated by nano-reversed phase HPLC (Agilent 1100; flow split by a tee to  $\sim 100$   $\text{nl}/\text{min}$ ) and introduced into either an ETD-enabled LTQ quadrupole linear ion trap (Thermo Fisher Scientific, San Jose, CA) or LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer via nano-ESI. Samples analyzed using the LTQ were run in a data-dependent manner with the five most abundant species subjected to both ETD and collision-induced dissociation fragmentation (in separate alternating scans). Ion trap instrumental parameters were used as described recently (24). LTQ-Orbitrap analyses were performed according to parameters presented previously (25). Targeted analyses were also performed with the LTQ-Orbitrap where target  $m/z$  values of interest, which were observed in CE-MS analyses but were not characterized previously, were inspected. Spectral data were converted into .dta files (DTAs) using Bioworks Browser and MakeDTA (a gift from Don Hunt) for collision-induced dissociation and ETD, respectively. Collision-induced dissociation and ETD DTAs were subsequently made into separate batches of  $\sim 2000$  files each.

Alternatively LC-MALDI-TOF and LC-Q-TOF peptide sequencing was performed as described in detail elsewhere (26).

**Identification of Peptide Sequences**—MS/MS data were submitted to MASCOT (Matrix Science) for a search against human entries in the Mass Spectrometry Protein Sequence Database (MSDB). Accepted parent ion mass deviation was 50 ppm; accepted fragment ion mass deviation was 500 ppm. All search results with a MASCOT peptide score better than 20, depending on the ion coverage as related to the main spectra features, were accepted. Data files were also searched against the National Center for Biotechnology Information (NCBI) human non-redundant database using the Open Mass Spectrometry Search Algorithm (OMSSA) with an E-value cutoff of 0.01. The number of basic and neutral polar amino acids of obtained peptide sequences was utilized to correlate peptide sequencing data to CE-MS data as described earlier (26).

**Statistical Analyses**—All of the statistical analyses for patient characteristics and clinical data were performed using the Minitab (Minitab for Windows 12.21, Minitab Inc., State College, PA) software package. The Kolmogorov-Smirnov test was used to test for normal distribution of the data. Data are expressed as mean  $\pm$  S.D. if normally distributed or median (interquartile range) if their distribution was not normal. Categorical variables are presented as frequency counts, and intergroup comparisons were analyzed by  $\chi^2$  test for smoking, gender, and medication with statins. For continuous variables, differences between the groups were evaluated using unpaired Student's  $t$  test or Mann-Whitney  $U$  test for variables that were normally distributed and those that were not normally distributed, respectively.

Estimates of sensitivity and specificity were calculated based on tabulating the number of correctly classified samples. Confidence intervals (95% CI) were calculated in MedCalc (MedCalc for Windows 8.1.1.0, Medcalc Software, Mariakerke, Belgium). The ROC plot was obtained by plotting all sensitivity values (true positive fraction) on the y axis against their equivalent ( $1 - \text{specificity}$ ) values (false positive fraction) for all available thresholds on the x axis (MedCalc Software). The AUC was evaluated because it provides a single measure of overall accuracy that is not dependent upon a particular threshold (20).

## RESULTS

Details of patients and controls are given in Table I. Control subjects had lower blood pressure and C-reactive protein levels compared with CAD patients. Due to treatment with statins total cholesterol and LDL cholesterol levels were lower in patients with CAD than in healthy controls. However, control subjects had higher HDL cholesterol levels and were more likely to have never been smokers compared with patients with CAD. Renal function was similar in both groups. Compared with control subjects the augmentation index was higher, and large and small artery elasticity indices were lower in patients with CAD.

At base-line evaluation, midstream urine specimens were available from 86 of 88 patients; nine of 86 urine samples did not fulfill quality control criteria (15). Of the remaining 77 samples, 30 were used to establish a training set, and 47 were evaluated in a blinded assessment (Table II). The 77 patients who entered analysis did not differ in clinical characteristics from the 88 originally included in the study. In addition to healthy controls from the same population (Glasgow, UK), a group of healthy controls from another population (Hannover, Germany) and patients before and after treatment with ramipril (Nürnberg, Germany) were used to rule out center-specific bias and effect of medication on constituting markers,

TABLE I  
Demographics and clinical data

eGFR, estimated glomerular filtration rate; AI, augmentation index; C1, large artery elasticity index; C2, small artery elasticity index; BMI, body mass index. *p* values are given for the comparison between CAD at base line and CAD at follow-up. Significant differences between controls and CAD at base line are indicated. Values in parentheses are interquartile range. N/A, not applicable.

	Controls ( <i>n</i> = 32)	CAD, base line ( <i>n</i> = 77)	CAD, follow-up ( <i>n</i> = 82)	<i>p</i>
Sex, male/female	21/9	56/21	59/23	0.91
Age (yr)	54 ± 13	61 ± 11 †	62 ± 11	N/A
BMI (kg/m <sup>2</sup> )	25.3 ± 3.1	26.2 ± 4.8	26.5 ± 4.6	0.38
Smokers, active/stopped/none	3/9/20	14/38/25 <sup>a</sup>	13/42/27	0.93
Diabetic, <i>n</i>	0	6	7	0.84
Statin therapy, <i>n</i>	0	75 <sup>b</sup>	77	0.93
Systolic blood pressure (mm Hg)	123 ± 12	132 ± 20 <sup>c</sup>	133 ± 19	0.42
Diastolic blood pressure (mm Hg)	76 ± 7	76 ± 9	74 ± 9	0.15
Total cholesterol (mmol/liter)	5.4 ± 0.9	3.9 ± 0.8 <sup>b</sup>	3.8 ± 0.8	0.24
LDL cholesterol (mmol/liter)	3.2 ± 0.7	1.9 ± 0.7 <sup>b</sup>	1.8 ± 0.8	0.13
HDL cholesterol (mmol/liter)	1.5 ± 0.4	1.2 ± 0.3 <sup>c</sup>	1.3 ± 0.3	0.12
Triglycerides (mmol/liter)	1.3 (1.0;2.7)	1.5 (1.8; 2.2)	1.4 (1.1; 2.1)	0.48
C-reactive protein (mg/liter)	1.3 (0.3;2.4)	2.6 (1.0; 6.3) <sup>c</sup>	1.2 (0.6; 2.2)	<0.001
Creatinine (μmol/liter)	90 ± 9	91 ± 21	98 ± 23	0.03
eGFR (ml/min/1.73m <sup>2</sup> )	75 ± 10	75 ± 9	69 ± 14	<0.001
AI (%)	26.4 ± 11.7	32.1 ± 10.4 <sup>a</sup>	30.5 ± 9.4	0.18
C1 (ml/mm Hg × 10)	14.0 ± 3.9	11.8 ± 4.3 <sup>c</sup>	13.3 ± 4.3	0.01
C2 (ml/mm Hg × 100)	5.7 ± 3.9	3.8 ± 2.8 <sup>c</sup>	3.6 ± 1.6	0.50

<sup>a</sup> *p* < 0.05.

<sup>b</sup> *p* < 0.001.

<sup>c</sup> *p* < 0.01.

TABLE II  
Characteristics of the training and the test sets

BMI, body mass index. Values in parentheses are interquartile range.

	Training set				Test set	
	CAD <sup>a</sup>	Controls <sup>a</sup>	Ramipril samples	Hannover samples	CAD <sup>a</sup>	Controls <sup>a</sup>
Total number of patients	30	20	18	232	47	12
Sex, male/female	22/8	14/6	14/4	101/137	34/13	7/5
Age (yr)	62 ± 11	54 ± 13	59 ± 11	34 ± 11	61 ± 12	54 ± 12
BMI (kg/m <sup>2</sup> )	25.9 ± 3.8	25.7 ± 3.5	30.5 ± 5.0		26.5 ± 5.3	24.8 ± 2.2
Smoker, yes/no	5/25	2/18	4/14		8/39	1/11
Diabetic, <i>n</i>	1	0	18		5	0
Systolic blood pressure (mm Hg)	133 ± 19	123 ± 11	151 ± 13		132 ± 20	124 ± 13
Diastolic blood pressure (mm Hg)	75 ± 11	76 ± 7	86 ± 10		76 ± 8	75 ± 8
Total cholesterol (mmol/liter)	3.9 ± 0.8	5.2 ± 1.0	5.5 ± 1.3		3.9 ± 0.9	5.6 ± 0.6
LDL cholesterol (mmol/liter)	1.9 ± 0.6	3.0 ± 0.8	3.4 ± 1.0		1.9 ± 0.8	3.4 ± 0.6
HDL cholesterol (mmol/liter)	1.2 ± 0.3	1.4 ± 0.4	1.3 ± 0.3		1.2 ± 0.3	1.6 ± 0.4
Triglycerides (mmol/liter)	1.6 (1.2; 2.4)	1.3 (1.1; 2.5)	1.6 (1.0; 2.7)		1.4 (1.2; 1.9)	1.3 (1.0; 1.6)

<sup>a</sup> In all depicted parameters there were no differences between CAD patients and controls in the training set and test set, respectively.

respectively (Table II). At follow-up evaluation only five of the 82 urine samples failed quality control criteria.

Samples were analyzed by CE-MS, and biomarker peptide patterns distinguishing CAD from healthy controls could be established (Fig. 1 and Table III). The discriminatory ability of the classification factor *F* to distinguish CAD and healthy controls in the training set was assessed using ROC analysis and showed an AUC of 0.96 (Fig. 2A). A classification threshold,  $F_{CAD} = 13.0$ , classified the training set with sensitivity of 97% (95% CI, 83–99) and specificity of 80% (95% CI, 56–94). Furthermore a test set including patients after coronary artery

bypass graft surgery and patients after acute coronary syndrome was subject to a blinded study. Of 59 urine samples 48 scored positive as “CAD,” and 11 scored negative as healthy controls using the threshold of  $F_{CAD} = 13.0$ . After unblinding, 46 of 47 CAD samples and 10 of 12 healthy control samples were predicted correctly (Fig. 2C) with sensitivity of 98% (95% CI, 89–99) and specificity of 83% (95% CI, 52–97). A ROC analysis showed an AUC of 0.94 (Fig. 2B).

We used a fixed classification factor threshold ( $F_{CAD} = 13.0$ ) to calculate sensitivity and specificity and were able to generate a risk profile for patients having CAD. This is demon-

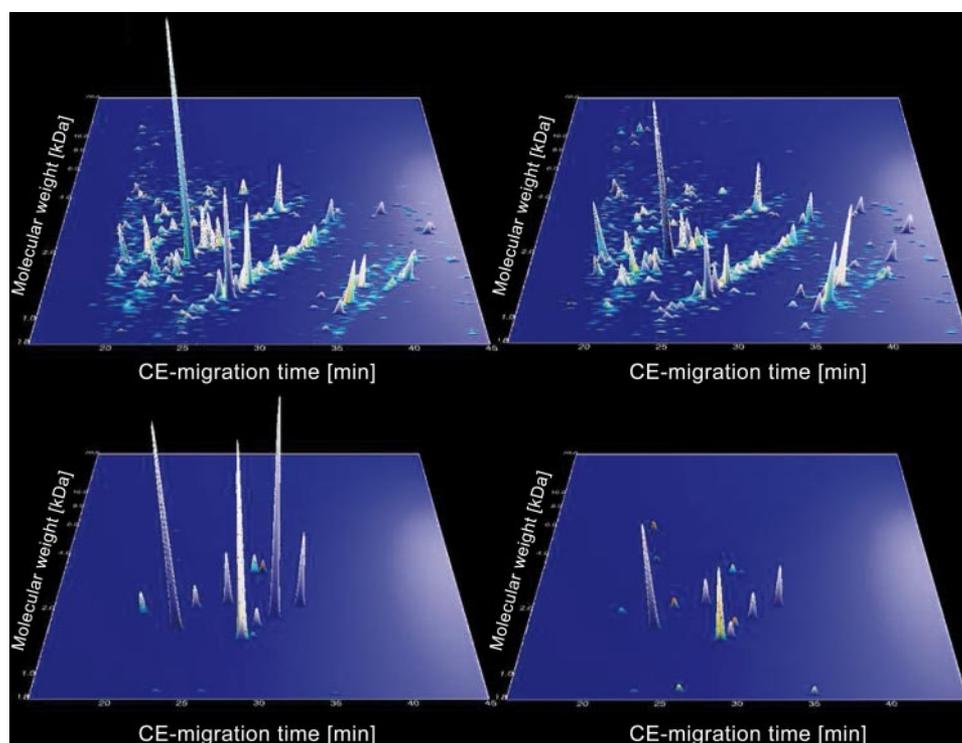


FIG. 1. **Polypeptide patterns distinguishing patients with CAD from controls.** This figure shows the compiled data sets of 30 CAD samples (*upper left panel*) and 20 control subjects (*upper right panel*) of the training set (Table II). Normalized molecular weight is plotted against normalized migration time. The mean signal intensity is given in three-dimensional depiction. The *lower panels* depict the 15 indicative polypeptides defining the specific pattern for CAD (*lower left panel*) and controls (*lower right panel*).

TABLE III  
CAD polypeptide panel

Polypeptide markers derived from the training set of 30 CAD patients and 20 healthy controls (supplemental table) are shown. ID, polypeptide ID annotated by the SQL database as described under "Experimental Procedures"; mass, molecular mass (Da); migration time, normalized to an array of 200 polypeptides frequently found in urine. HC, healthy controls; IQR, interquartile range.

Polypeptide identification			CAD		HC		<i>p</i> value
Protein ID	Mass	Migration time	Median	IQR	Median	IQR	
	<i>Da</i>	<i>min</i>					
629	838.44	35.05	0.0	74.6	151.4	259.2	0.0003
948	858.43	23.26	20.0	95.8	112.2	156.9	0.0469
994	860.40	26.23	0.0	39347.0	149.0	269.8	0.0006
16954	1435.72	28.86	6167.6	3515.3	2896.3	2872.6	0.0111
18225	1487.71	29.58	344.6	560.9	803.6	673.8	0.0459
21244	1623.8	24.15	7087.0	4054.0	4074.9	3193.8	0.0017
22223	1664.82	29.87	891.3	359.5	443.0	658.5	0.0019
25791	1834.9	31.15	5309.2	9423.3	1205.1	924.4	0.0018
27916	1933.95	21.63	785.7	677.4	207.2	341.3	0.0026
30362	2056.02	25.44	1047.1	687.7	555.9	444.9	0.0005
31262	2104.04	33.04	2985.6	1463.6	1951.9	1117.4	0.0174
32138	2150.04	27.76	2344.2	1112.7	1429.7	1277.5	0.0023
52939	3137.51	30.32	602.6	547.1	154.9	303.8	0.0001
53283	3158.60	26.69	767.8	1133.9	182.0	753.8	0.0052
81912	5574.44	23.22	238.4	489.3	568.9	346.6	0.0442

strated by the significant differences between *F* values obtained in patients with CAD *versus* healthy controls. The mean *F* value for all CAD samples ( $n = 47$ ) was  $16.55 \pm 2.0$ , whereas that for control samples ( $n = 12$ ) was  $9.04 \pm 4.8$  ( $p < 0.001$ ) (Fig. 2C). The calculated *F* value and the resulting *F* value for each patient's urine sample can be used to predict the risk for CAD. The

probability of CAD at a given classification factor *F* taking into account the related probability for no disease was calculated, and  $p_{CAD}$  was plotted against the obtained *F* values (Fig. 2D).

To test whether this assessment can also be used to evaluate the effects of therapeutic interventions, we analyzed patients who had undertaken different levels of physical ex-

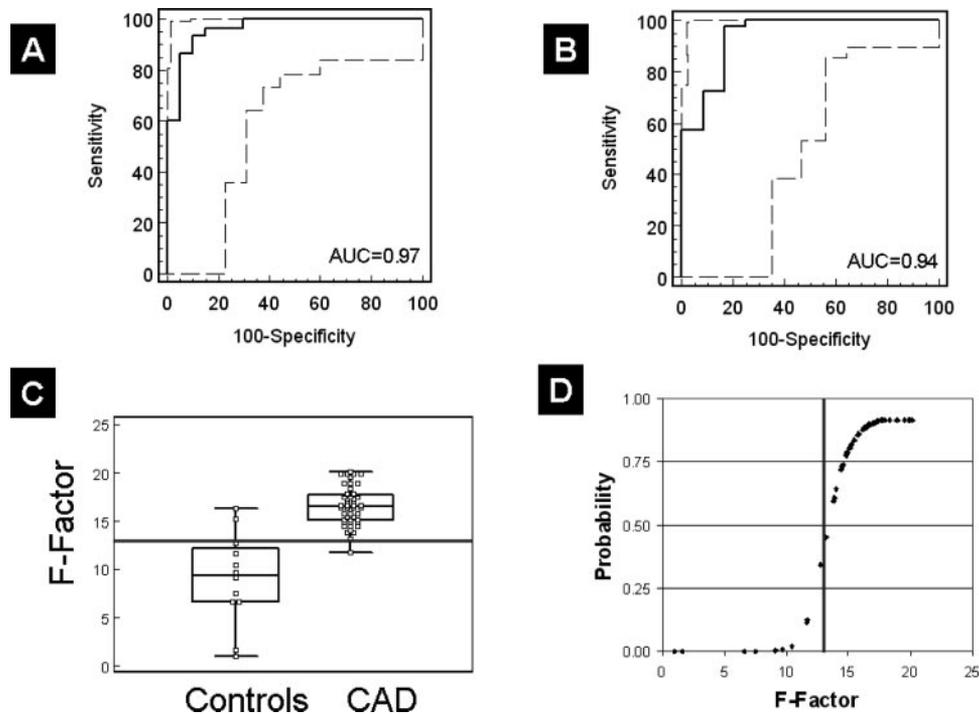


FIG. 2. *A* and *B*, ROC curves of the proteomics panel diagnosis. Using the CAD-specific polypeptide panel from Table III the classification factor  $F$  is used as a variable in ROC analysis in the 50 samples of the training set (CAD and controls in Table II, *bold line*,  $AUC = 0.97$ ) (*A*) and in the 59 samples of the test set (CAD and controls in Table II, *bold line*,  $AUC = 0.94$ ) (*B*). 95% CIs are indicated by *thin lines*. *C*, box-and-whisker plots of classification factor  $F$  obtained for classification of the test set (Table II). The *boxes* depict the quartiles  $Q_1$  and  $Q_3$  of each distribution; the statistical medians are shown as *horizontal lines* in the *boxes*. The *whiskers* indicate 3 and 2 times the interquartile range of  $Q_1$  and  $Q_3$ , respectively. *D*, CAD probabilities of the 59 urine samples of the test set are plotted against the classification factor  $F = \sum_i c_i \log A_i$ .

ercise. Self-reported and physiotherapist-validated activity levels determined improvement of the classification factor between base-line and follow-up assessment. At follow-up assessment the classification factor was almost unchanged in inactive and low grade active patients ( $\Delta F = -0.20$ ), whereas the very active patient group ( $\Delta F = -1.90$ ) showed a significant improvement ( $p = 0.02$ ).

To determine the identity of biomarkers in the panel (Table III) used to distinguish CAD from healthy controls we performed LC-MS/MS to obtain sequence information. Examples of sequences deduced from high resolution fragmentation spectra are shown in Fig. 3, and five sequences of biomarkers in the panel could be identified: collagen  $\alpha$ -1(I) chains and collagen  $\alpha$ -1(III) fragments (Table IV). In all cases, the identified collagen type I or III fragments were up-regulated in CAD samples compared with controls. From the initial marker list 38 polypeptides (supplemental table) could be identified. The majority of these sequences were collagen fragments.

#### DISCUSSION

The aim of our study was to establish and validate a proteome-based non-invasive method for the detection and follow up of CAD. Therefore, we tested whether CE-MS can resolve signature patterns of urinary polypeptides (15) that can be used as biomarkers.

Our polypeptide pattern distinguished between the pres-

ence and absence of disease. Furthermore we were also able to demonstrate a dynamic behavior of the polypeptide pattern in response to exercise. Inactive patients had no change in pattern over time, whereas very active patients showed significant changes toward a “healthier” biomarker pattern. These results illustrate an important difference between proteomics *versus* genomics analysis. Genomics analysis identifies predisposing risk factors, whereas proteomics can identify the point in time when predisposition is developing into disease. This is because the proteome is inherently dynamic and hence can better reflect changes (27). This also pertains to measuring the effects of therapeutic interventions and will assist in personalized medicine strategies. Furthermore this observation also provides a link between the kidney and CAD on a molecular basis and may help to explain why chronic kidney disease is one of the best predictors for CAD (28).

For a reliable CAD-specific polypeptide panel it is mandatory that the constituting markers are independent of medication effects because the majority of patients require multiple drugs including antihypertensives. As an example, we used an additional control group of patients before and after treatment with an angiotensin-converting enzyme inhibitor, ramipril, to refine selected polypeptides. This comparison offered a more precise and sensitive monitoring of medication effect that is independent of individual fluctuations in urinary

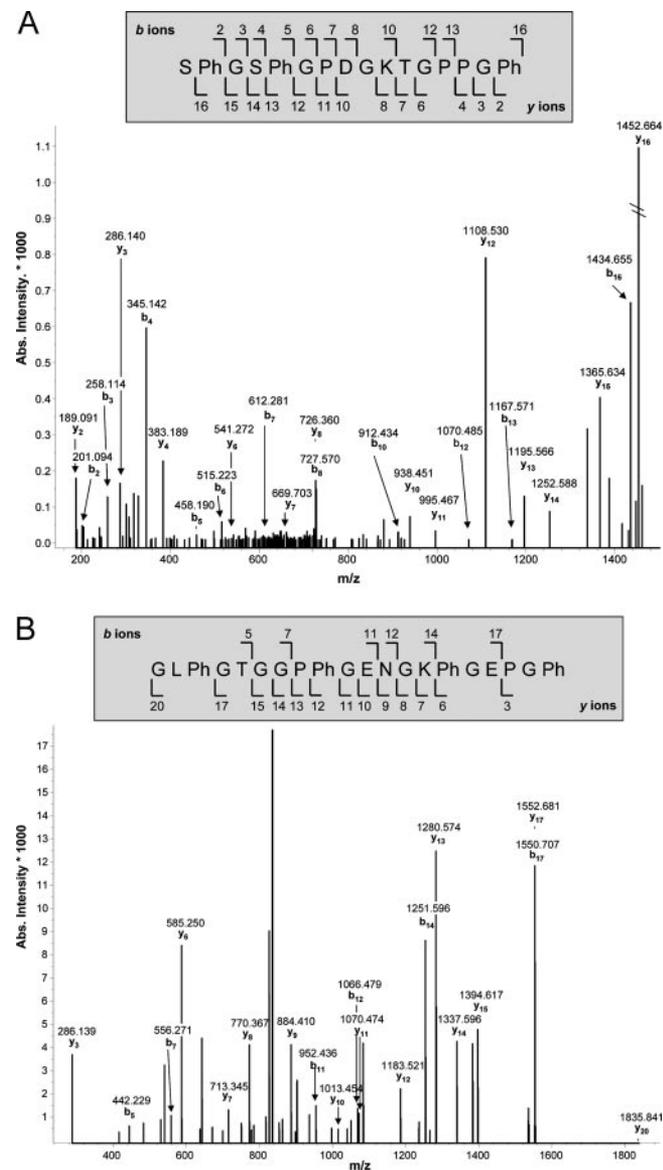


FIG. 3. A, high resolution MS/MS spectrum (lower panel) of 1435.72-Da polypeptide indicative for CAD (Table III). This peptide was found to be up-regulated in CAD samples compared with control samples (Table I). Swiss-Prot database matching indicated this is a fragment of collagen  $\alpha$ -1(I) chain (amino acids 543–558) (*Homo sapiens*) with a calculated mass of 1435.72 Da and a sequence as indicated (upper panel). B, MS/MS spectrum (lower panel) of 1834.90-Da polypeptide (Table III) up-regulated in CAD samples (Table I). Database matching indicated this is a fragment of collagen  $\alpha$ -1(III) chain (amino acids 642–661) (*H. sapiens*) with a calculated mass of 1834.83 Da and a sequence as indicated (upper panel). The masses of b-ion fragments and y-ion fragments are correlated with the obtained sequence using fragment numbers (52, 53). Abs. Int., absolute intensity.

peptide patterns. The obtained polypeptide pattern was validated using a test set including patients with CAD in addition to healthy controls.

We were also able to identify five of the polypeptides constituting the CAD-specific panel: all of them were collagen

type I or III fragments. These collagens are predominant proteins in the arterial walls. They also appear together in the thickened intima of atherosclerotic lesions (29). Collagen is synthesized containing C-terminal and N-terminal propeptide sequences (PICP and PINP for type I procollagen and PIICP and PIINP for type III procollagen). These propeptides are used as biomarkers of the rate of collagen synthesis (PICP), collagen degradation (ICTP), and collagen turnover (PIIINP) (30–34). In the normal artery, both synthesis and degradation of extracellular matrix proteins are remarkably slow. Atherosclerosis leads to increased synthesis of many matrix components, including collagen types I and III, elastin, and several proteoglycans (35).

All sequenced collagen fragments were up-regulated in CAD samples compared with controls. In line with these sequence data suggesting elevated collagen degradation levels, increased circulating levels of collagenases, such as MMP-9, have been reported in patients with stable angiographic coronary atherosclerosis (36, 37) or intermittent claudication (38). In patients with stable CAD, circulating MMP-9 levels independently predict rapid lumen diameter reduction (39) and fatal cardiovascular events (40). Elevated MMP-9 activity has been found in unstable plaques, suggesting a crucial role in plaque rupture (41, 42).

The majority of the identified polypeptides constituting the initial marker list were also collagen fragments (supplemental table). These findings suggest that CAD-specific information is redundantly available in urine samples in the form of different detectable collagen fragments. In addition to the collagen fragments, a fragment of membrane-associated progesterone receptor component 1 was identified (supplemental table). Progesterone receptors are reported to be associated with thoracic ascending aorta, internal carotid artery, coronary artery, and left atrial appendage (43).

Several groups have reported on the application of proteomics techniques to analyze tissue or plaque specimens to study cardiovascular disease or arteriosclerosis (44–48). Although these results provide new insights into disease-related pathways, they do not allow non-invasive detection of coronary artery disease. Only a few of these studies focused on the analysis of body fluids derived through minimally invasive means. Furthermore either these studies were based on pooled blood specimens (49, 50), making individual sample classification impossible, or they included only small patient cohorts without blinded studies for validation (51). All of these limitations are avoided in our study. The proteomics analysis of urine allowed for the reproducible and standardized analysis of a non-invasively obtained body fluid for highly accurate detection of CAD, which was subsequently validated in a blinded study.

In summary, in this patient population a CAD-specific urinary proteome panel is an accurate and non-invasive predictor for CAD. These and other similar biomarkers have the potential to be used for early diagnosis and thus more efficient prophylaxis as well as monitoring of therapeutic interventions

TABLE IV  
Sequence data

aa, amino acids; Ph, hydroxyproline.

Polypeptide identification			Sequence information			
Protein ID <sup>a</sup>	Experimental mass	Migration time	Sequence	Name	Calculated mass	Mass deviation
	Da	min			Da	ppm
16954	1435.72	28.86	SPhGSPGPDGKTGPPhGP	Collagen $\alpha$ -1(I) chain (aa 543–558) ( <i>H. sapiens</i> )	1435.66	43
21244	1623.80	24.15	DGAPhGKNGERGGPhGGPhGP	Collagen $\alpha$ -1(III) chain (aa 587–604) ( <i>H. sapiens</i> )	1623.72	41
25791	1834.90	31.15	GLPhGTGGPPhGENGKPhGEPGPh	Collagen $\alpha$ -1(III) chain (aa 642–661) ( <i>H. sapiens</i> )	1834.83	34
27916	1933.95	21.63	GDDGEAGKPGRPhGERGPPPhGP	Collagen $\alpha$ -1(I) chain (aa 230–249) ( <i>H. sapiens</i> )	1933.89	34
53283	3158.60	29.69	GERGSPhGGPhGAAGFPhGARGLP-hPhPGSNGNPgPPPhGPh	Collagen $\alpha$ -1(III) chain (aa 861–895) ( <i>H. sapiens</i> )	3158.44	51

<sup>a</sup> Sequence data obtained from polypeptides in Table III.

and as novel drug targets.

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