# Evaluation of Urine Proteome Pattern Analysis for Its Potential To Reflect Coronary Artery Atherosclerosis in Symptomatic Patients

**ARTICLE** *in* JOURNAL OF PROTEOME RESEARCH · DECEMBER 2008

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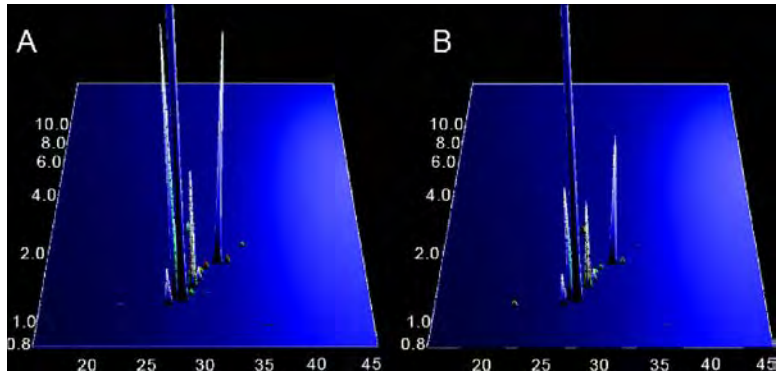


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# Evaluation of Urine Proteome Pattern Analysis for Its Potential To Reflect Coronary Artery Atherosclerosis in Symptomatic Patients

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**Received August 10, 2008**

Coronary artery disease (CAD) is a major cause of mortality and morbidity. Noninvasive proteome analysis could guide clinical evaluation and early/preventive treatment. Under routine clinical conditions, urine of 67 patients presenting with symptoms suspicious for CAD were analyzed by capillary electrophoresis directly coupled with mass spectrometry (CE-MS). All patients were subjected to coronary angiography and either assigned to a CAD or non-CAD group. A training set of 29 patients was used to establish CAD and non-CAD-associated proteome patterns of plasma as well as urine. Significant discriminatory power was achieved in urine but not in plasma. Therefore, urine proteomic analysis of further 38 patients was performed in a blinded study. A combination of 17 urinary polypeptides allowed separation of both groups in the test set with a sensitivity of 81%, a specificity of 92%, and an accuracy of 84%. Sequencing of urinary marker peptides identified fragments of collagen α1 (I and III), which we furthermore demonstrated to be expressed in atherosclerotic plaques of human aorta. In conclusion, specific CE-MS polypeptide patterns in urine were associated with significant CAD in patients with angina-typical symptoms. These promising findings need to be further evaluated in regard to reliability of a urine-based screening method with the potential of improving the diagnostic approaches for CAD.

**Keywords:** Coronary artery disease • atherosclerosis • urine • proteome • peptide pattern

## Introduction

Atherosclerotic cardiovascular disease is a leading cause of morbidity and mortality in industrialized countries.[1] Early diagnosis of coronary artery disease (CAD) would provide the opportunity to institute a preventive lifestyle, pharmacological treatments, and even measures such as prophylactic stenting, aiming to effectively reduce the ultimate risk of developing cardiac ischemia and myocardial infarction.[2] The benefits of currently available noninvasive screening methods are restricted by limitations in specificity, sensitivity, availability and cost. Troponins have been proven to be highly reliable markers of acute cardiac ischemia. Additionally, plasma proteins such as C-reactive protein (CRP) have been found to be associated with plaque inflammation and increased risk for cardiovascular events.[3] Unfortunately, in many cases, only coronary angiography allows for the confident diagnosis or exclusion of CAD. However, based on the risks associated with this invasive procedure and its costs, coronary angiography is typically regarded as the final step of CAD diagnosis pinpointing the urgent need for reliable, noninvasive biomarkers for CAD.

Proteomic technologies emerged as sensitive, fast and robust tools for analysis of protein patterns in body fluids.[4,5] Compared to conventional process of marker definition, this approach allows inclusion of large numbers of proteins to identify diagnostically useful protein patterns. Accompanied by advanced software, proteomic analysis enables the de novo establishment of protein patterns without any prior definition of proteins of interest. Proteomic analysis has been performed using various technology platforms, mainly two-dimensional gel electrophoresis or high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS).[6] An alternative method directly coupling capillary electrophoresis (CE) and MS (CE-MS), running with small sample volumes and high sensitivity, provides a unique tool for clinically usable proteomic analysis.[5,6] In particular, urine has been mostly used for these

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10.1021/pr800615t CCC: $40.75 

proteomic analyses, since it is found to be stable against proteolytic degradation, contains a low concentration of irrelevant proteins, and can be collected noninvasively.[7,8]

With the sue of CE-MS, we recently performed a pilot study comparing proteomic patterns in urine of patients with various degrees of symptomatic and nonsymptomatic angiographically confirmed CAD to a control group of younger, mostly healthy volunteers, who although not angiographically proven were clinically free of signs of CAD.[9] This study indicated that CAD might be associated with specific changes in the urine proteome. We now use a similar approach to investigate whether CE-MS derived urine or plasma polypeptide patterns could be used for the diagnosis of CAD in patients who are presenting with clinical symptoms suspicious for CAD. This directly represents the group of patients and the clinical routine setting, where such a proposed proteomic diagnostic test would be performed. While plasma samples were not stable when not immediately processed by CE/MS, urine proteome analysis resulted in reliable patterns even after longer storage times. In a rigorous statistical approach, we restricted the investigation to patients with the clear diagnosis or exclusion of CAD by coronary angiography, which is considered to be the gold standard for the diagnosis of CAD. We were able to identify a urine polypeptide pattern that is associated with CAD and therefore warrants further evaluation as a diagnostic test for the workup of patients with symptoms suspicious for CAD. Furthermore, we were able to confirm by histology that the identified peptides are derived from specific forms of collagens that we could identify in specimens of human atherosclerotic plaques. This emphasizes the pathophysiological relevance of our findings.

## Material and Methods

**Patients, Plasma/Urine Sampling and Definition of Coronary Artery Disease.** Samples of patients referred for coronary angiography due to new-onset or unstable angina were investigated with the aim to identify CAD-associated polypeptide patterns using CE-MS. Included were patients older than 18 years with angina-typical symptoms combined with classical risk factors for atherosclerotic disease (hyperlipidemia, diabetes mellitus, history of smoking, positive family history of CAD, hypertension). Patients with renal disease, acute coronary syndromes with positive troponin I/T or significant CK/CK-MB elevation, acute inflammatory diseases, cardiogenic shock, congestive heart failure, significant calcific valve disease, atherosclerotic aneurysms, previous history of peripheral artery disease and known cerebrovascular disease were excluded from the training as well as test set. Blood samples were taken via vein puncture and immediately centrifuged at 4 °C, and heparin-plasma was isolated and stored at −80 °C. Spot urine samples were obtained using urine monovettes (Sarstedt, Nuembrecht, Germany) and immediately stored at −80 °C. Body fluid sampling was coordinated and controlled with standard operation procedures to minimize data corruption by sampling biases. Written informed consent was obtained from all participants according to the voting of the ethics committees of the University of Freiburg, Germany.

After performing coronary angiography, patients without evidence of atherosclerosis were defined as the control group ("non-CAD"), whereas patients with two or three vessel-disease and lesions of at least 75% stenosis were defined as the CAD group. A first set of 29 patients was used for the establishment of a CAD-associated proteomic pattern ("training set"), which was then applied to a second, independent set of 38 patients ("test set"). The design of this study is according to current guidelines for studies on clinical proteomics[10] and the minimum information about proteomics experiments (MIAPE).[11]

**Sample Preparation. Urine.** For CE-MS analysis, 0.7 mL aliquots of urine samples were thawed immediately before use and diluted with 0.7 mL of 2 M urea, 10 mM $NH_4OH$ containing 0.02% SDS. To remove high-abundance proteins, the samples were filtered using Centrisart ultracentrifugation filters (20 kDa MWCO) at 3000 rpm until 1.1 mL of filtrate was obtained. The filtrate was desalted using PD-10 column (Amersham Bioscience, Uppsala, Sweden), equilibrated in 0.01% $NH_4OH$ in HPLC-grade in $H_2O$ (Roth). After lyophilization, all samples were stored at 4 °C and suspended in HPLC-grade $H_2O$ shortly before analysis.

**Plasma.** For proteomic analysis, 0.7 mL aliquots of the −80 °C frozen samples were thawed immediately before delipidation.[12] Therefore, 0.7 mL of heparin-plasma was added to 0.7 mL *n*-butanol/*iso*-proyl ether 4:6 (v/v), mixed, and centrifuged in a benchtop centrifuge at 14 000 rpm at 4 °C for 10 min. A three-phase system was obtained with a yellow organic supernatant, a gray, highly viscous interphase and a clear, yellow plasma phase that was collected. For ultrafiltration, 0.5 mL of delipidated plasma was diluted with 0.5 mL of 8 M urea, followed by 1 mL of $H_2O$, and filtered using Centrisart ultracentrifugation filters (10 kDa MWCO; Sartorius) at 3000 rpm until 1.4 mL of filtrate was obtained. Using 20 kDa cutoff filters resulted in incomplete removal of high-abundance plasma polypeptides, such as IgG light chains. Therefore, cutoff was shifted from 20 to 10 kDa in comparison to the processing of the urine samples. The filtrate was then applied to a PD-10 desalting column (Amersham Bioscience, Uppsala, Sweden), equilibrated in 0.01% $NH_4OH$ in HPLC-grade in $H_2O$ (Roth, Germany). The column was washed with 1.6 mL of ammonium buffer and eluted with 2 mL of ammonium buffer. Finally, all samples were lyophilized, stored at 4 °C and suspended in HPLC-grade $H_2O$ shortly before analysis, as described.[7]

**Histology of Human Atherosclerotic Plaques.** Human healthy vessels and plaque materials from fibro-fatty lesions were obtained from autopsy with the approval of the Ethics Committee of the Cardiology Research Industrial Complex, Moscow. All samples were embedded in OCT (TissueTek, Sakura) and cut into 6 $\mu$m thick serial sections by cryosection. Frozen sections were thawed at room temperature for 30 min and then fixed in acetone at −20 °C for 20 min. Samples were sequentially fixed again in 10% buffered formalin at room temperature for 2 min and absolute ethanol for 10 min to reduce background. Subsequently, all slides were treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Slides were blocked with normal serum for 30 min, avidin for 15 min and biotin for 15 min, respectively. After the blocking steps, the slides were treated with either anti-collagen antibodies or normal serum as negative control for 1 h at room temperature. Both anti-collagen type I (clone 2150−0020) and anti-collagen type III (clone 2150−0100) antibodies were purchased from AbD Serotec, Oxford, U.K., and were diluted by 1:10. Subsequently, all samples were incubated with anti-rabbit biotinylated secondary antibody for 30 min, following incubation with avidin−biotin-peroxidase system (Vectastain ABC Kit, Vector). The reaction products were stained with a DAB substrate kit for peroxidase (Vector) to give a brown reaction product. Finally, the slides were counterstained with hematoxylin and mounted.

**CE-MS Analysis and Quality Control.** CE-MS analysis was performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) coupled online with a Micro-TOF MS (Bruker Daltonic, Germany).[7] The ESI sprayer (Agilent Technologies) was grounded, and the ion spray interface potential was set between −4.0 and −4.5 kV. Data acquisition and MS acquisition methods were automatically controlled by the CE via contact-close-relays. Spectra were accumulated every 3 s, over a range of $m/z$ 350−3000. For urine, the average recovery of the sample preparation procedure was approximately 85% with a detection limit of ∼1 fmol.[7] For ion peaks with charges $z \leq 6$, monoisotopic mass signals were resolved resulting in mass accuracy of <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks ($z > 6$). Repeatability for analyzing the same replicate and reproducibility achieved for repeated processing of the same urine sample was assessed. The 200 most abundant polypeptides ("internal standard" peptides) could be detected with a rate of 98%. Post preparation stability of the analytical system was assessed with consecutive measurements of the same replicate over a period of 24 h. No significant loss of peptides and proteins was observed implying the stability of the CE-MS setup, the postpreparative stability of the urine samples at 4 °C and their resistance to oxidizing processes or precipitation.[7,13]

Data were accepted only if the following quality control criteria were met: a minimum of 950 peptides/proteins for urine samples and 430 peptides/proteins for plasma samples must be detected with a minimal MS resolution of 8000 (required resolution of peaks with $z = 6$) in a minimal migration time interval (the time window, in which separated peptides can be detected) of 10 min for urine samples and 6 min for plasma samples. After calibration, the deviation of migration time must be below 0.35 min. Samples that did not fulfill one of these four criteria were reanalyzed.

**Data Processing and Cluster Analysis.** Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software.[7] Only signals observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. The software employs a probabilistic clustering algorithm and uses both isotopic distributions as well as conjugated masses for charge-state determination of peptides/proteins. MosaiquesVisu automatically eliminates all signals that can be detected only as singly charged species. The resulting peak list characterizes each protein/peptide by its molecular mass and its migration time. Subsequently, mass, migration time and ion signal intensity (amplitude) were normalized with respect to internal "housekeeping peptides"[7] to correct for analytical intersample differences and naturally varying urine concentrations. These housekeeping peptides are present in all urine samples so far analyzed in frequencies >90% and with small relative amplitude standard deviations. All detected polypeptides were deposited and annotated in a Microsoft SQL database, allowing further analysis and digital compilation of multiple samples. For clustering processes, 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 Supplementary Table in Supporting Information.

**Statistical Methods.** All of the statistical analyses for patient characteristics and clinical data were performed using Microsoft Excel. Sensitivity and specificity were calculated based on tabulating the number of correctly and falsely classified samples. Confidence intervals (95% CI) and the Receiver Operating Characteristic (ROC) plot were obtained using MedCalc (MedCalc for Windows 8.1.1.0, Medcalc Software, Mariakerke, Belgium). The area under the ROC curve (AUC) was evaluated as it provides a single measure of overall accuracy that is not dependent upon a particular threshold.[14]

**Definition of Biomarkers and Sample Classification.** For biomarker definition, polypeptides found in more than 70% of the samples in at least one of the two groups (CAD or non-CAD) were considered. This predefined set of polypeptides was further validated by randomly excluding 30% of available samples. This bootstrapping procedure was repeated up to 10 times. Further on, nonparametric methods such as Whitney-Mann test (rank sum test) were applied for selection refinement. Obtained discriminating polypeptides with $P < 0.05$ as significance level were analyzed using ROC statistics. The obtained AUC values were used as ranking parameter to define best predictors. The obtained markers were further validated by comparing the 11 CAD samples with 99 age-matched samples from healthy volunteers without any known CAD history (Mosaiques Diagnostics database) to minimize statistical artifacts. Model establishment and sample classification was 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 observed amplitude of marker $i$.

**Sequencing of Polypeptides.** Candidate biomarkers and other native peptides from urine were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail.[15] In addition, peptide sequencing was performed on a Dionex Ultimate 3000 nanoflow system connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source. Chromatographic separation of peptides was performed using reversed-phase C18 nanocolumn (NanoSeparations, Nieuwkoop, Netherlands) and a linear gradient (60 min) from 2−50% acetonitrile in 0.1% aqueous formic acid. MS was operated in data-dependent mode and automatically switched between MS and MS/MS acquisition. Survey full scan MS spectra (from $m/z$ 300−2000) were acquired with resolution 60 000 at $m/z$ 400. The most intense ions were sequentially isolated for fragmentation using a linear ion trap, collision-induced dissociation and detection in either the linear ion trap or in the Orbitrap. Orbitrap MS/MS were acquired with resolution 15.000 at $m/z$ 400. General mass spectrometric conditions were electrospray voltage 1.6 kV; no sheath or auxiliary gas flow; ion transfer tube temperature, 225 °C; collision gas pressure, 1.3 mT; normalized collision energy, 32% for MS. Ion selection threshold was 500 counts for MS/MS. In addition to using the Oribtrap, MS/MS experiments using a Bruker HCT$^{ultra}$ ion trap (Bruker Daltonics, Bremen, Germany) were performed. For this purpose, large proteins were removed from neat urine by filtering through a 20 kDa nanosep filter (Pall corporation, MI) prior to loading an aliquot of 150 $\mu$L on a 4.6 mm Eclipse XDB-$C_{18}$-5 $\mu$m column (Agilent Technologies, CA). The column was eluted into 10 × 1 mL fractions by a linear gradient of acetonitrile (5−50%) in 0.1% TFA(aq) using an Agilent HPLC system. Following drying, the samples were redissolved in 20 $\mu$L (0.1% formic acid(aq)) and loaded on an Agilent Zorbax SB-$C_{18}$-5 $\mu$m column (150 mm × 0.5 mm) The column was eluted by a linear gradient (2−44% acetonitrile in 0.1% formic acid(aq)) using a capillary LC (Agilent Technologies) connected to a Bruker HCT$^{ultra}$ ion trap. Peptide sequences were determined from MS/MS spectra

recorded by preset targeted masses obtained from CE-MS experiments.

All resultant MS/MS data were submitted to MASCOT (www.matrixscience.com) for a search against human entries in the Swiss-Prot database 55.1 (359 942 sequences; 129 199 355 residues). The following parameters were used for the search: methionine oxidation, proline oxidation, and lysine oxidation (variable modification); maximal missed cleavages, 0; enzyme, none. Accepted parent ion mass deviation, accepted fragment ion mass deviation, and scores are listed in Table 2. Only search results with a MASCOT peptide score of 25 or higher, which also met ion coverage stipulations as related to the main spectral features, were included. The number of basic and neutral polar amino acids of the peptide sequences was utilized to correlate peptide sequencing data to CE-MS data, as described earlier.

## Results

**Dependency of Plasma Polypeptide Patterns on Storage Time and Procedure.** Kolch et al.[10] demonstrated that protein degradation is a major disadvantage in the use of serum for proteomic analysis and suggested plasma as a possible alternative. To investigate plasma stability under various handling and storage conditions, a fresh plasma pool from a 30 year old, healthy volunteer was split into three different samples. One sample was prepared immediately, a second one was prepared after storage for 2 h at 4 °C, and a third one analyzed after an additional freeze/thaw step. All samples were analyzed in three replicates. The obtained results document an increasing number of CE-MS signals due to the storage of the samples for 2 h at 4 °C, which could not be observed for urine (Figure 1). This effect was further enhanced by the indispensable freezing step that is a prerequisite for long time sample storage and shipment. Preparation of urine following either the urine protocol or the plasma protocol including a delipidation step resulted in highly comparable urine profiles (data not shown), excluding the protocol itself as origin of additional signals. Thus, special focus on constant sample processing and strict analytical quality controls is prerequisite to optimize plasma data consistency.

Two groups of patients not differing in basic characteristics, medication, or clinical presentation but clearly classified by angiography to a group with severe CAD ($n = 15$) or to a group with angiographically normal coronary arteries ($n = 14$) were evaluated for differences in proteome patterns. For all plasma samples analyzed by CE-MS, a mean of 710 ± 281 polypeptides could be detected in a 10.1 ± 3.9 min time window (raw data migration time interval). Calibration of mass, migration time and signal amplitude was performed as described in detail elsewhere.[7] After calibration, a mean migration time interval (the time window, in which separated polypeptides can be detected) of 12.3 ± 2.1 min related to a mean deviation of migration time (dT) of 0.23 ± 13 min was obtained. To ensure good data comparability, samples that did not fulfill one of these four criteria were prepared and analyzed again. Of the 29 plasma samples in the training set, 8 did not fulfill these quality control criteria after repeated measurements/preparations and were not used for further evaluation. Thus, to define potential CAD-specific biomarkers in plasma, 11 samples from patients suffering from CAD were compared to 10 samples from individuals without any evidence of CAD in angiography (Figure 2 and Supplementary Table 2 in Supporting Information). To enable discrimination between these two groups, 39 plasma

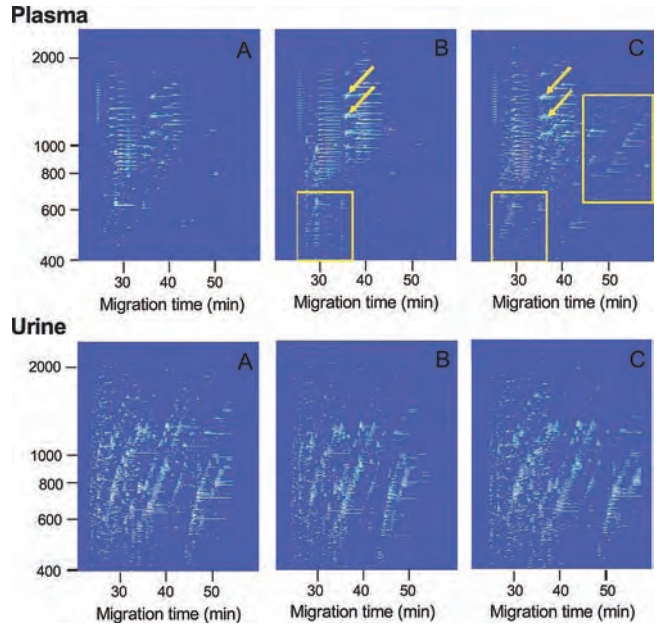


**Figure 1.** CE-MS raw data demonstrate dependency on storage conditions for plasma and urine proteome. Fresh (A) plasma (upper panel), plasma stored at 4 °C for 2 h (B) and plasma stored at 4 °C for 2 h followed by freezing/thawing (C) are compared to the respective results for urine (lower panel). Ratio *m/z* is plotted against CE-migration time in minutes. Signals observed due to storage conditions are marked with yellow boxes. The yellow arrows indicate a remarkable signal broadening upon storage for plasma samples. This suggests several similar degradation fragments of high molecular weight proteins, slightly varying in molecular weight and CE-migration time, absent in the fresh sample. Comparable effects could not be found for urine samples confirming earlier observations.[7,8]

polypeptides (Supplementary Table 2 in Supporting Information) had to be combined to a polypeptide panel. Although the discriminatory ability to distinguish CAD and controls in the training set was characterized by sensitivity 90.9% [95% CI 58.7−98.5] and specificity 90.0% [95% CI 55.5−98.3] using a classification threshold $F_{CAD}$ of −0.70, attempts to validate these findings in a blinded assessment failed. This is most likely due to overfitting and, in comparison to urine, lower reproducibility of the low molecular weight plasma proteome/peptidome. As a consequence of this apparent failure to validate the potential biomarkers, no further efforts were undertaken to obtain sequence from these peptides.

**Urinary Polypeptide Profiling and Reproducibility of Polypeptide Patterns.** The suitability of urine for stable polypeptide profiling was assessed by the reproducibility achieved for repeated preparation, including repeated freezing/thawing steps, and measurement of the same urine sample (Figure 1). The 200 most abundant polypeptides were repeatedly 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 stability of urine samples and their resistance to, for example, oxidizing processes or precipitation. These data are in accordance with recent reports demonstrating the stability and advantages of urine for proteome analysis.[7,8] For

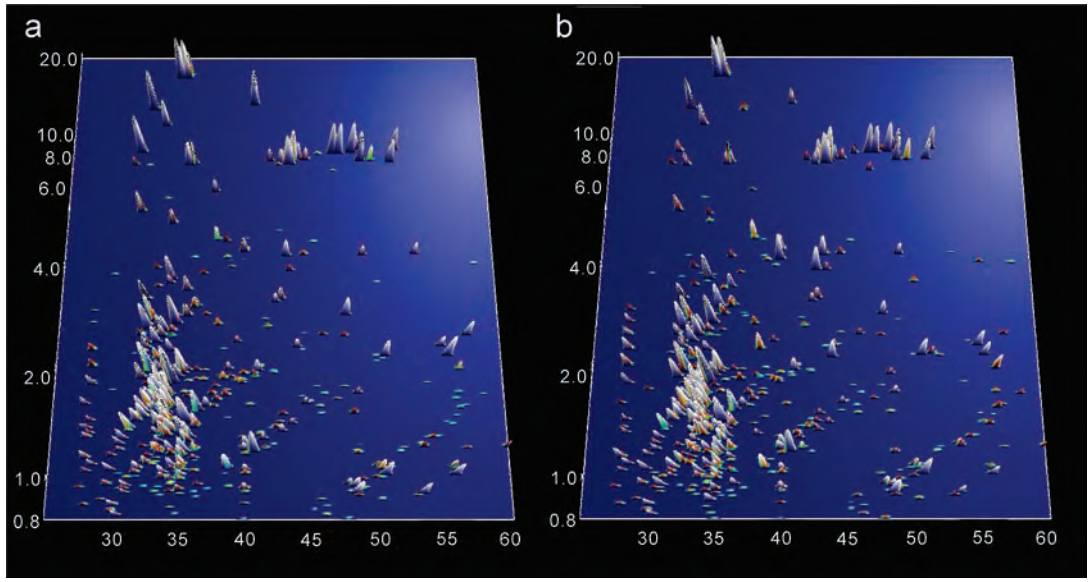


**Figure 2.** Compiled plasma CE-MS data of patients suffering from CAD (a) versus controls (b). Molecular mass (kDa) is plotted against CE-migration time in minutes. The average MS signal intensity of 662 different polypeptides is color-coded.

**Table 1.** Patient Characteristics in the Training and Test Sets

(A) Training Set

| | CAD ($n$ = 15) | no CAD ($n$ = 14) | $P$-value |
|---|---|---|---|
| Age [years] | 61.1 | 59.6 | n.s. |
| Sex [male/female] | 11/4 (73% male) | 10/4 (71% male) | n.s. |
| smokers [$n$] | 9 (60%) | 7 (50%) | n.s. |
| diabetic [$n$] | 2 (13%) | 1 (7%) | n.s. |
| hyperlipidemia [$n$] | 7 (47%) | 6 (43%) | n.s. |
| Hypertension [$n$] | 13 (87%) | 12 (86%) | n.s. |
| pos. family history for CAD | 6 (40%) | 5 (36%) | n.s. |
| creatinine [mg/dL] | 1.02 | 0.96 | n.s. |

(B) Test Set

| | CAD ($n$ = 26) | no CAD ($n$ = 12) | $P$-value |
|---|---|---|---|
| age [years] | 68.4 | 65.6 | n.s. |
| sex [male/female] | 20/6 (77% male) | 7/5 (59% male) | n.s. |
| smokers [$n$] | 13 (50%) | 5 (42%) | n.s. |
| diabetic [$n$] | 10 (38%) | 3 (25%) | n.s. |
| hyperlipidemia [$n$] | 8 (31%) | 4 (33%) | n.s. |
| hypertension [$n$] | 20 (77% | 8 (67%) | n.s. |
| pos. family history for CAD | 4 (15%) | 3 (25%) | n.s. |
| creatinine [mg/dl] | 1.08 | 0.99 | n.s. |

these reasons, we focused on the assessment of CAD-associated polypeptide patterns in urine.

**Establishment of Acute CAD-Associated Pattern in Urine.** In the training set, a group of patients with two- or three vessel CAD ($n$ = 15) was compared to a group of patients with angiographically normal coronary arteries (non-CAD, $n$ = 14). Typical clinical cardiovascular risk factors and CRP were not different between the two patient groups (Table 1A). Of the 29 urine samples, 5 samples of CAD patients did not fulfill the quality control criteria[7] necessary for the establishment of a training set and were not used for further analysis. Out of 5856 polypeptides, 163 proteins were identified in urine that were present in at least 70% of one group, with differences in frequency of >40%, differences in amplitude >1.4 and resisting initial bootstrapping steps. On the basis of nonparametric rank sum tests, from these initial markers, a set of 17 polypeptides was combined in a diagnostic panel indicating the presence of CAD (Figure 3, Figure 4 and Supplementary Table in Supporting Information). Using this pattern, our training set revealed a sensitivity of 100% [95% CI 71−100] and a specificity of 93% [95% CI 66−99] using a classification threshold $F_{CAD}$ of 33.8 for the diagnosis of coronary artery disease (Figure 4, left panel).

**Validation of the Acute CAD-Associated Pattern in a Blinded Study ("Test Set").** As suggested for clinical proteomic studies,[16] these established biomarkers were validated in a blinded study to control the established machine learning model for potential overfitting: a potential pitfall in multiparametric classification in clinical proteomics.[17] A total of 38 patients with clinical presentations as defined above and identical baseline characteristics (Table 1B) were classified following coronary angiography, and spot urine samples were analyzed in a blinded fashion. No urine samples had to be excluded from this analysis due to quality issues. With the use of the CAD-associated model, 22 samples scored for the CAD and 16 for the non-CAD pattern. After unblinding, 21/26 CAD patients and 11/12 controls were correctly identified as having, or not having CAD (Figure 4). The obtained sensitivity of 81% [95% CI 60−93], the specificity of 92% [95% CI 62−99], the accuracy of classification with 84%, and the difference in the area under the ROC curve compared to guessing probability ($p$ < 0.0001) argue for the potential of the presented strategy to be used as a noninvasive screening test for CAD.

**Application of the CAD-Associated Urine Polypeptide Pattern to Further Patient Groups.** The CAD-associated panel was applied to a randomly selected independent set of 120 urine samples of patients with bladder cancer ($n$ = 20), prostate cancer ($n$ = 20), macro- ($n$ = 20), micro- ($n$ = 20), normalbuminuric diabetes mellitus ($n$ = 20), and renal failure ($n$ = 20) without any known CAD history (patient details on Mosaiques Diagnostics database and Supplementary Table in Supporting Information). While for patients with malignancies 85% were negative for the CAD-associated polypeptide pattern, approximately 50% of the patients with renal failure were positive for the CAD-associated pattern. This suggests that chronic renal failure causes a massive disturbance of the urine

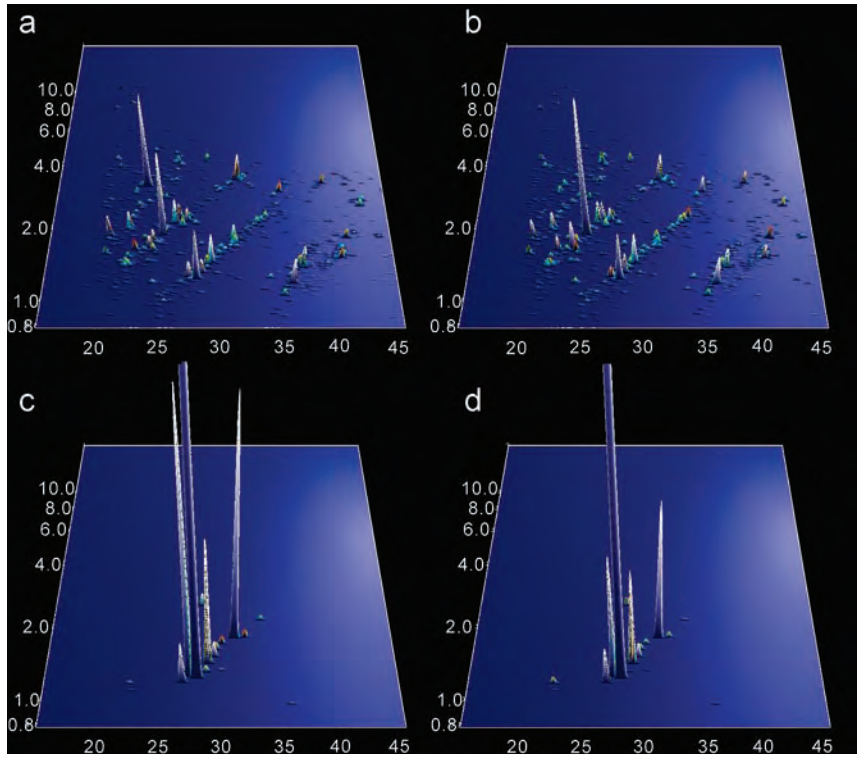


**Figure 3.** Three-dimensional depiction of the urinary CAD-associated polypeptide patterns. Molecular mass in kDa (*y*-axis) is plotted against CE-migration time in minutes (*x*-axis). The average MS signal intensity of the markers is plotted in the *z*-direction. The compiled serum CE-MS data of more than 3000 polypeptides are given for CAD (a) and non-CAD patients (b). A differentiation pattern of 17 polypeptides was established and compared for CAD (c) and non-CAD (d) patients. A majority of the observed signal amplitudes are up-regulated in the CAD patients.

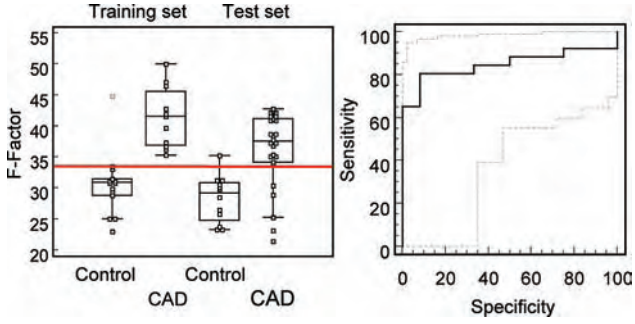


**Figure 4.** (Left Panel) Box-and-whisker plots of the classification factor *F* obtained for 24 spot urine samples from CAD patients ($n = 11$) and control subjects ($n = 13$) of the training set and the test set in a blinded assessment of urine samples from CAD patients ($n = 26$) and control subjects ($n = 12$). 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 3/2 times the interquartile range of $Q_1$ and $Q_3$. Red horizontal line depicts classification threshold $F_{CAD} = 33.8$. (Right Panel) ROC curve of 38 samples of the test set using the proteomics panel approach (bold, AUC = 0.85). Observed sensitivity and specificity of the classification (threshold $F_{CAD} = 33.8$) is 81% and 92%, respectively. Dashed curve represents 95% confidence intervals.

proteome that may interfere with a CAD-specific urine proteome analysis in these patients.

**Identification and Histological Validation of CAD-Associated Urine Polypeptides.** We were able to sequence eight out of the 17 polypeptides, which proved to be indicative for CAD within the urine proteome (see Supplementary Table in Supporting Information; four representative MS/MS spectra are depicted in Figure 5). These high-resolution fragmentation spectra could be matched to the following Swiss-Prot database entries: Collagen α-1(I) [543−558], [543−559] [546−558], [798−810], [800−810], [1042−1071], and Collagen α-1(III) [642−661], [796−808] (Table 2). In all cases, the identified peptides were up-regulated in CAD samples compared to controls.

To validate the findings, we immunostained human aortic tissue samples obtained by autopsy for collagen type I and III. In contrast to normal aortic wall tissue, atherosclerotic plaques strongly stained for collagen type I and III (Figure 6) supporting the observed up-regulation of collagen I and III fragments in CE-MS analysis of CAD-patients compared to controls.

## Discussion

The major finding of this study is the reflection of coronary artery disease (CAD) in specific polypeptide patterns of the urine proteome, using samples of patients from a "real world" clinical setting. Urine samples of 24 patients with symptoms suspicious for CAD were angiographically classified into a CAD and a non-CAD group. These groups were used to establish CAD-associated polypeptide patterns in urine. The novel method of direct-coupled capillary electrophoresis and mass spectrometry (CE-MS) combined with a tailored bioinformatics software package allowed this unique proteomic pattern analysis. In a blinded approach, the established polypeptide pattern was tested in 38 patients, all presenting with angina-typical symptoms. The established urine proteome pattern allowed the identification of CAD or non-CAD patients with high sensitivity, specificity and accuracy.

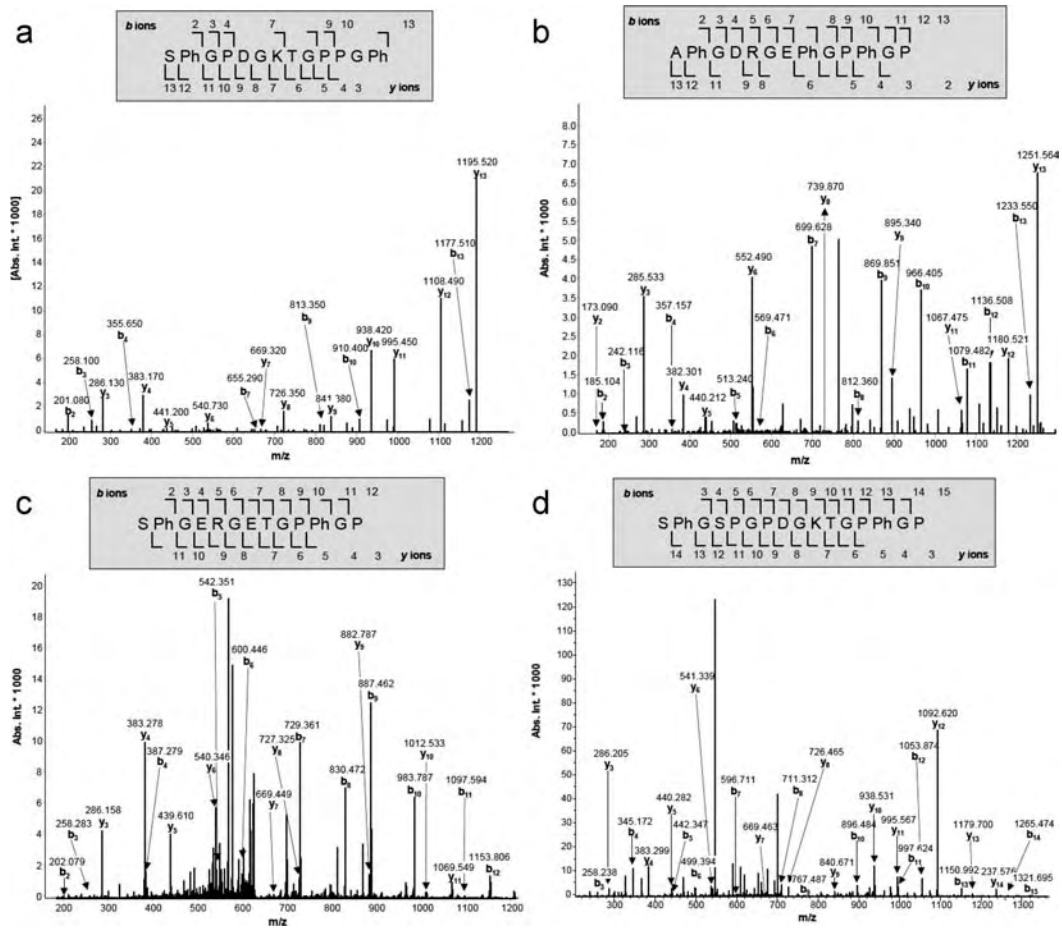


**Figure 5.** Four exemplary high-resolution MS/MS spectra of the polypeptides (Table 2), which could be identified in the urine proteome as being upregulated in CAD samples compared to control samples (Table 1). Swiss-Prot database matching indicated these as human collagen fragments with the calculated masses given in Table 2 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.

An important prerequisite for a clinically useful test is its feasibility under clinical settings, which includes variations in sampling and storage conditions. The use of plasma for proteomic analysis has the major limitation that plasma exhibits a high protease activity and that the plasma proteome is dependent on storage conditions (Figure 1). In contrast, urine has been described as a stable matrix for proteome analysis, as urine can be stored for several hours at room temperature or for long-term at −80 °C;[7,8] in the current study, we were able to confirm this finding in a direct comparison between plasma and urine samples (Figure 1). Indeed, all urine samples from 38 patients in the test set could be analyzed by CE-MS. No specimens in the test set had to be excluded due to quality issues, which is an important factor for potential clinical applications.

In line with a study recently published by Zimmerli et al.,[9] we demonstrate the feasibility of a urine proteome approach for the diagnosis of CAD. However, in the study described here, we were able to establish an improved proteome pattern under routine clinical conditions, reflecting CAD in the clinically important set of patients presenting with CAD-suspicious symptoms and comparable cardiovascular risk factors. This is a very important prerequisite for clinical application of such a polypeptide pattern, as such a test needs to identify CAD-associated symptoms with high sensitivity and specificity, facilitating the noninvasive triage of patients.

Zimmerli et al.[9] compared a cohort of 88 patients scheduled for coronary artery bypass grafting to a group of healthy volunteers also using the combination of CE-MS as used in the present study. Out of 187 biomarkers identified in this study, 84 were also identified in our study.[9] This similarity is remarkable considering the differences in patient groups between the two studies. Indeed, the comparison in the polypeptide patterns obtained in these two separate studies strongly supports our major conclusion that CAD is reflected in specific polypeptide patterns in urine. CE-MS has been successfully used previously for biomarker discovery in diverse clinical settings, such as the development of disease-specific biomarkers for renal diseases[18] or various cancers.[7,19] Additionally, stage-specific markers for various diseases[20,21] have allowed the ability to prognosticate in various disease settings.[22]

The described proteomic analysis fulfils several criteria required for a successful multibiomarker approach for the diagnosis of CAD as recently proposed:[23] (1) *Reproducibility* of CE-MS with urine samples has been demonstrated in our studies using repeated measurements as well as in several recently published reports.[7,13,24] (2) *Sensitivity and specificity* of the described urine polypeptide patterns to classify a patient's CAD status in the test set was 82% and 92%, respectively. These statistical parameters came out stronger than results obtained in a recent study of serum proteome analysis for the detection of venous thromboembolism by MS,

**Table 2.** Sequence Data Obtained from Urinary Polypeptides[a]

| polypeptide identification | | | sequence information | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| protein-ID* | experimental mass [Da] | migration time[13] | sequence | name | calculated mass [Da] | mass deviation [ppm] | observed precursor mass/charge | peptide mass tolerance | fragment mass tolerance | Mascot score |
| 9438 | 1186.591 | 22.27 | DDGEAGKPhGRPhG | collagen α-1 (I) [800−810] | 1186.521 | 59 | 594.2651/2+ | ±5 ppm | ±0.7 Da | 71 |
| 9666 | 1194.602 | 26.73 | SPhGPDGKTGPPGPh | collagen α-1 (I) [546−558] | 1194.552 | 42 | 598.2600/2+ | ±0.05 Da | ±0.1 Da | 68 |
| 11446 | 1250.613 | 27.94 | APhGDRGEPhGPPhGP | collagen α-1 (I) [798−810] | 1250.553 | 48 | 626.3409/2+ | ±0.15 Da | ±0.7 Da | 56 |
| 12132 | 1268.621 | 27.33 | SPhGERGETGPPhGP | collagen α-1 (III) [796−808] | 1268.563 | 46 | 635.4019/2+ | ±0.3 Da | ±0.7 Da | 41 |
| 16954 | 1435.719 | 28.86 | SPhGSPGPDGKTGPPhGP | collagen α-1 (I) [543−558] | 1435.658 | 43 | 718.9034/2+ | ±0.2 Da | ±0.7 Da | 76 |
| 19003 | 1522.760 | 29.39 | SPhGSPhGPDGKTGPPGPhA | collagen α-1 (I) [543−559] | 1522.690 | 49 | 762.4384/2+ | ±0.2 Da | ±0.7 Da | 63 |
| 25791 | 1834.895 | 31.15 | GLPhGTGGPPhGENGKPhGEPGPh | collagen α-1 (III) [642−661] | 1834.833 | 34 | 918.4242/2+ | ±5 ppm | ±0.1 Da | 72 |
| 41423 | 2583.299 | 28.31 | AGPPhGAPhGAPGPVGPAGKSGDRGETGP | collagen α-1 (I) [1042−1071] | 2583.231 | 26 | 862.6555/3+ | ±2 Da | ±0.3 Da | 29 |

[a] On the left side, this table contains the polypeptide identification parameter of the CE-MS analysis (Protein-ID, experimental mass [Da], and CE-migration time). On the right side, sequence information derived from MS/MS analysis are depicted. The amino acid sequences are listed beginning with the N-terminus of the peptide. In addition, the protein fragment name (peptide coverage based on Swiss-Prot entry) and the theoretical mass [Da] are given. Furthermore, mass deviation [ppm] of the theoretical and the measured CE-MS mass are shown. In the last columns, the observed precursor mass and charge derived from the MS/MS experiment and different parameters of the Mascot database searches (peptide and fragment mass tolerance, and Mascot score) are listed.

which in itself proved to be more reliable than D-dimer testing but only resulted in sensitivities and specificities of 68% and 89%, respectively.[25] Furthermore, in our study the analysis of a large cohort of 261 healthy individuals in the collective urine proteome database of Mosaiques Diagnostics only revealed a positive score <15% for the 17 selected polypeptides. When applying the pattern to an independent set of 60 patients with diabetes mellitus with various degrees of functional impairment of the kidneys and 40 patients with cancer, the percentage of patients demonstrating atherosclerosis-associated patterns reached values consistent with the expected CAD prevalence in theses groups. Particularly in patients with chronic kidney disease, a high prevalence of CAD is to be expected.[26−28] (3) The *cost* of CE-MS is expected to be in a range that will permit screening for CAD on a large-scale basis. (4) The *potential impact on medical care* of a noninvasive urine test for the detection of atherosclerotic disease in particular of the coronary arteries may be highly significant for early diagnosis and early treatment of CAD.

Our study population includes a control group of patients with angina-typical symptoms, comorbidities and atherosclerosis-associated risk factors. Nevertheless, our pattern analysis was able to exclude CAD in 11/12 patients of the test set with angiographically nondiseased vessels in spite of the presence of risk factors and angina-typical symptoms. Thus, our proteomic approach seems to be robust enough to detect or exclude atherosclerotic disease of the coronary arteries in patients who typically present to chest pain units or emergency departments for diagnosis or exclusion of CAD.

Only a small number of other studies focusing on proteomic analysis of atherosclerosis have been published. With the use of two-dimensional gel-electrophoresis, Duran et al.[29] found a markedly increased number of protein spots in the supernatant of cultivated human endaterectomy-specimens compared to normal artery segments. In another original proteomic study, the proteome of aortas from apolipoprotein E −/− mice at different stages of atherogenesis was studied and highlighted associations of immune-inflammatory responses, oxidative stress and energy metabolism.[30]

In contrast to urine, the reproducibility of proteome analysis in plasma may be limited by high protease activity[13] (Figure 1). To reduce this impact, fast processing of plasma samples and strict standard operating procedures to address these potential variances were employed in this study. From sampling, immediate cooling on ice until centrifugation, and storage at −80 °C, a maximal time course of 1 h was ensured, which may certainly be an enormous challenge in routine clinical testing. The preparation procedure had to be optimized by including a delipidation step,[12] since many samples showed very high blood lipid concentration preventing ultrafiltration. An important step to ensure high data consistency was the establishment of strict quality control criteria. Despite all these efforts, 39 peptides (Supplementary Table 2 in Supporting Information) that displayed highest discriminatory abilities assessed by ROC analysis ranking had to be combined in a panel to distinguish CAD patients from controls with machine learning procedures. The majority of these polypeptides did not reach a significance level below 0.05 in nonparametric statistics. Additionally, the number of polypeptide dimensions exceeds the number of patient dimensions in the machine learning model, resulting necessarily in model overfitting and clearly disqualifying the model for further validation. In summary, these results implicate further demands for investigations

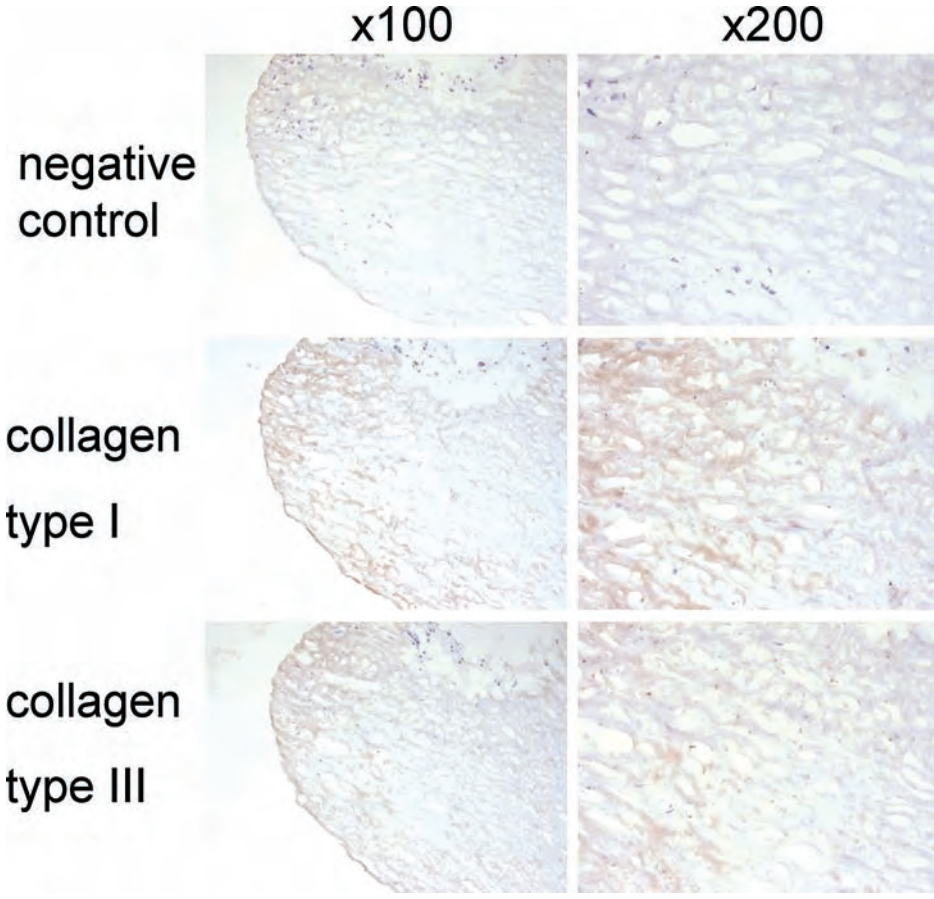


**Figure 6.** Immunohistology of collagen type I and III in human atherosclerotic plaques. Collagen type I and III staining is positive in comparison to nonspecific antibody control. The 100- and 200-fold magnifications are shown from a representative histological preparation done with samples of three patients.

to deal with the (in)stability of the plasma proteome in proteomics experiments in general and particularly in clinical settings. Urine represents the ultrafiltrate of plasma and may thus reflect proteomic changes of biomarkers relatively reliably in a broad range of diseases. However, urine may not be usable as the optimal body fluid for biomarker discovery for all diseases. In particular in diseases that are associated with changes in renal function, plasma may be preferable to urine for biomarker discovery.

Whereas a diagnostic pattern itself contains the central diagnostic information, sequence analysis of individual peptides may provide further insights in mechanisms of atherogenesis. Although using state-of-the-art tandem mass spectrometry and despite major efforts, we were not able to sequence all of the CAD pattern-constituting polypeptides. However, we may have reached the technical limits of technology available nowadays in top-down sequencing of naturally occurring peptides.[31] In general, native peptide sequencing is limited by post-translational modifications, complicating not only peptide fragmentation, but also subsequent database searches.[31,32] In addition, the applied CE-MS technology is able to identify polypeptides with a high analytical sensitivity,[7,33] whereas tandem mass spectrometry used for sequencing has higher detection limits.[34,35]

The most abundant polypeptides in the urine pattern associated with CAD originated from collagen. This finding points toward a central role of collagen in the pathophysiology of atherosclerosis. Our histological findings, demonstrating an increased expression of collagen type I and III in human atherosclerotic plaques, support this conclusion. There are also several publications suggesting that collagen is of central importance in atherosclerosis. In fibrous human plaques, collagen types I and III were described as the prevalent structural elements.[36,37] Also in lipid-rich, rupture-prone atherosclerotic plaques from patients with carotid artery stenosis, morphologically diverse collagen type I and III-positive structures were identified. These were furthermore shown to stimulate platelet adhesion and thrombus formation in vitro.[38] In a very recent imaging study of laser scanning microscopy of atherosclerotic plaques in mice, collagen type I and III were clearly identified in plaques of ApoE-mice, but not in healthy arteries.[39] Those studies suggest a pathogenic role of collagen type I and III in the development and progression of atherosclerotic plaques as well as in the formation of thrombi on fibrous and lipid-rich plaques. Also, elevated levels of collagenases such as MMP-9 have been described in patients with atherosclerotic disease and various risk profiles.[40−42] None of the diseases investigated so far with CE-MS revealed a specific urinary secretion of collagen fragments as found in patients with CAD, suggesting a CAD-specific collagen increase and degradation.[7,20] Overall, our findings together with literature reports, demonstrating an increase of specific collagen expression and an acceleration of specific collagen degradation in atherosclerosis, support the notion that the detection of collagen fragments in urine proteome indeed reflects atherosclerotic changes of the vasculature.

**Limitations.** The current pilot study is the first to establish polypeptide patterns that reflect coronary artery disease in patients with angina-typical symptoms. However, for establishing the described urine proteome analysis as a clinically applicable test, the effect of renal impairment on the established polypeptide pattern has to be further evaluated. So far, the urine polypeptide pattern associated with CAD has been found frequently in the group of patients with renal impairment. This is in accordance with the known high prevalence of CAD in these patients,[26−28] but may also be caused by the renal impairment itself. Also, these studies will have to evaluate whether the glomerular filtration rate, representing reduction of renal function, may influence the intensity of biomarker peaks. On the other hand, also plasma proteome profiling in such patients may be influenced by high concentrations of uremic toxins. In order to clarify this matter, further studies have to be performed in patients with renal impairment and angiographically defined coronary artery status.

In summary, in this study, a proteomic approach applied under routine clinical conditions using CE-MS in combination with a newly generated high-throughput software package supports the notion that CAD is reflected in specific polypeptide patterns in urine. Furthermore, peptides constituting these patterns can be used to identify proteins potentially important in atherosclerosis. Further studies are warranted to evaluate the potential clinical applicability of a urine-based proteome analysis for noninvasive diagnostic approaches in coronary artery disease.

**Acknowledgment.** We are grateful to Dr. Sheng Chen for technical assistance, to Dr. Belinda Smith for critical reading of the manuscript, and to Prof. Alex Bobik for providing human atherosclerotic plaque samples and advice in histological preparations. This work was funded by EU InGenious HyperCare LSHM-CT-2006-037093 to E.S., P.Z., A.F.D., and H.M. and the National Heart Foundation of Australia.

**Supporting Information Available:** Supplemetary tables and figures are referenced throughout the manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR800615T