Urine Proteome Analysis Reflects Atherosclerotic Disease in an ApoE<sup>−/−</sup> Mouse Model and Allows the Discovery of New Candidate Biomarkers in Mouse and Human Atherosclerosis*§

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Noninvasive diagnosis of atherosclerosis via single biomarkers has been attempted but remains elusive. However, a previous polymarker or pattern approach of urine polypeptides in humans reflected coronary artery disease with high accuracy. The aim of the current study is to use urine proteomics in ApoE<sup>−/−</sup> mice to discover proteins with pathophysiological roles in atherogenesis and to identify urinary polypeptide patterns reflecting early stages of atherosclerosis. Urine of ApoE<sup>−/−</sup> mice either on high fat diet (HFD) or chow diet was collected over 12 weeks; urine of wild type mice on HFD was used to exclude diet-related proteome changes. Capillary electrophoresis coupled to mass spectrometry (CE-MS) of samples identified 16 polypeptides specific for ApoE<sup>−/−</sup> mice on HFD. In a blinded test set, these polypeptides allowed identification of atherosclerosis at a sensitivity of 90% and specificity of 100%, as well as monitoring of disease progression. Sequencing of the discovered polypeptides identified fragments of α<sub>1</sub>-antitrypsin, epidermal growth factor (EGF), kidney androgen-regulated protein, and collagen. Using immunohistochemistry, α<sub>1</sub>-antitrypsin, EGF, and collagen type I were shown to be highly expressed in atherosclerotic plaques of ApoE<sup>−/−</sup> mice on HFD. Urinary excretion levels of collagen and α<sub>1</sub>-antitrypsin fragments also significantly correlated with intraplaque collagen and α<sub>1</sub>-antitrypsin content, mirroring plaque protein expression in the urine proteome. To provide further confirmation that the newly identified proteins are relevant in humans, the presence of collagen type I, α<sub>1</sub>-antitrypsin, and EGF was also confirmed in human atherosclerotic disease. Urine proteome analysis in mice exemplifies the potential of a novel multimarker approach for the noninvasive detection of atherosclerosis and monitoring of disease progression. Furthermore, this approach represents a novel discovery tool for the identification of proteins relevant in murine and human atherosclerosis and thus also defines potential novel therapeutic targets. *Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013847, 1–13, 2012.

Atherosclerosis and its complications, such as myocardial infarction or stroke, are a major health burden in industrialized countries and are about to become the leading cause of mortality and morbidity worldwide (1, 2). Atherosclerosis typically becomes manifest at a late stage with often fatal consequences such as myocardial infarction and stroke. Therefore, there is a large need for the detection of atherosclerotic disease before complications arise. However, the use of currently available noninvasive screening methods for atherosclerosis is limited by lack of specificity, sensitivity, availability, and cost (3, 4). Therefore, invasive procedures such as coronary angiography are often necessary for establishing a definitive diagnosis. Several biomarkers have been described with the potential to reflect the extent of atherosclerosis and the risk of atherosclerosis-driven complications, including adhesion molecules such as VCAM-1 (5, 6), CD40L (7), and hsCRP (8). The definitions of these makers have proven to be helpful for the understanding of the pathomechanisms of atherosclerosis, particularly its inflammatory component. However, they are less suitable to classify individual patients. A multimarker approach has been postulated to overcome these problems and to provide a promising approach to diagnose atherosclerosis (9).
Recently, we described a proteomic approach enabling the diagnosis of coronary artery disease (10, 11). Plasma and urine of patients with symptoms of angina were analyzed by capillary electrophoresis coupled to mass spectrometry (CE-MS)\(^1\) (12, 13). Because of high protease activity in plasma, a proteomic approach aiming to define a stable multimarker panel in plasma that would reflect atherosclerosis proved to be unsuccessful. However, in urine a proteome pattern could be established that reflected coronary artery disease in patients with an accuracy of 84%. Furthermore, sequencing of these markers revealed a pattern consisting of different collagen fragments (11).

Because the early stages of atherosclerotic disease in humans are difficult to detect and to quantify, we used the apolipoprotein E knock-out mouse model (ApoE\(^{-/-}\)) (14), as a well established animal model of atherosclerosis. Herein, urine proteome analysis by CE-MS proved to be a unique tool for discovery of novel biomarkers for early atherosclerotic disease, as well as for disease progression. Furthermore, we provide evidence for the biological relevance of the defined marker proteins in human atherosclerosis.

**EXPERIMENTAL PROCEDURES**

**ApoE\(^{-/-}\) Mouse Model and Urine Collection—**ApoE\(^{-/-}\) and C57BL/6 mice were obtained from the Baker IDI animal facility (Baker IDI Heart & Diabetes Institute, Melbourne, Australia). At the age of 6 weeks, the animals were divided into groups and either fed a high fat Western-type diet (high fat diet (HFD)) containing 21% fat and 0.15% cholesterol or a regular chow diet (CD). Altogether, this resulted in four groups with different treatments after the age of 6 weeks: ApoE\(^{-/-}\) on high fat diet (ApoE + HFD), ApoE\(^{-/-}\) on chow diet (ApoE + CD), and wild type C57BL/6 with either high fat or chow diet (WT + HFD and WT + CD). The care and use of laboratory animals followed the national guidelines and was approved by the institutional animal care and ethics committees of Baker IDI Heart & Diabetes Institute and the University of Freiburg.

**Urine Sample Collection, Procurement, and Preparation—**Urine sample collection was performed before the start of the diet described above in animals at the age of 6 weeks as base line (i.e. week 0). Thereafter, urine was repeatedly collected and evaluated at early (week 7) and late time points (week 12). For sampling, the mice were allocated into individual cages; each cage contained a water spillage tube, a food supply holder, and an air filter. Sterile plastic papers were placed to cover the bottom of the cage. To collect fresh urine, the investigators inspected each cage every 30 min for a 4-h period. Urine was collected and immediately stored at \(-20^\circ\)C. A 0.15-ml aliquot was thawed just before use and diluted with 0.15 ml of 2 M urea, 10 mM NH\(_4\)OH containing 0.02% SDS (15). To remove high molecular mass polypeptides, samples were filtered using Centrisart ultracentrifugation filter devices (20-kDa molecular mass cutoff; Sartorius, Gottingen, Germany) at 3,000 \(\times \) g until 0.2 ml of filtrate was obtained. Subsequently, filtrate was desalted using NAP5 column (GE Healthcare) equilibrated in 0.01% NH\(_4\)OH in HPLC grade water.

\(^1\) The abbreviations used are: CE, capillary electrophoresis; \(\alpha_\text{1}-\text{AT}\), \(\alpha_\text{1}-\text{antitrypsin}\); CD, chow diet; EGF, epidermal growth factor; HFD, high fat diet; MS/MS, tandem mass spectrometry; ROC, receiver operating characteristic; SVM, support vector machine; CI, confidence interval.
either converted into mgf files (RAW files generated by ion traps from Thermo Fisher Scientific) with the use of a data file generator (20, 21). All of the resultant MS/MS data were submitted to Mascot (www.matrixscience.com; release number 2.3.02) for a search against mouse entries (16,390 sequences) in the Swiss-Prot database (Swiss-Prot Number 2010.06) without any enzyme specificity and with up to one missed cleavage. No fixed modification was selected, and oxidation products of methionine, proline, and lysine residues were set as variable modifications. Accepted parent ion mass deviation was 2 ppm; accepted fragment ion mass deviation was 0.05 Da. Only search results with a Mascot peptide score equal to or higher than the Mascot score threshold were included (see Table II). Additionally, ion coverage was controlled to be related to main spectral fragment features (b/y or c/z ion series; supplemental Figs. 1–9). For further validation of obtained peptide identifications, the strict correlation between peptide charge at the working pH of 2 and CE migration time was utilized to minimize false positive identification rates (22); calculated CE migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared with the experimental migration time. The peptides were only accepted with a mass deviation below ±80 ppm and a CE migration time deviation below ±2 min.

Statistical Analysis—Sensitivity, specificity, and 95% confidence intervals (95% CI) were calculated using receiver operating characteristic (ROC) plots (23) (MedCalc version 8.1.1.0; MedCalc Software, Belgium, www.medcalc.be). The ROC plot was obtained by plotting all sensitivity values (true positive fraction) on the y axis against their equivalent (specificity) values (false positive fraction) for all available thresholds on the x axis. The area under the ROC curve was evaluated because it provides a single measure of overall accuracy independent of any threshold.

Peptides’ p values were calculated using the base 10 logarithm transformed intensities and the Gaussian approximation to the t-distribution. For multiple testing corrections, p values were corrected using the false discovery rate procedure introduced by Benjamini and Hochberg (24). The false discovery rate is the fraction of false positives among all tests declared significant. False discovery rate was controlled to be <0.05, which means that on average less than 5% of peptides declared significant are actually false positives. On the other hand, the other 95% of the biomarkers are indeed true positives. The approach is reported to have high statistical power for biomarker discovery in the situation of differential expression between two groups, when subjected to two different treatments, such as disease/no disease. Only proteins that were detected in a diagnostic group of subjects in at least 70% of samples were considered for testing. The test was implemented as macros in SAS (www.sas.com) and is part of the multitest R-package (www.biocductor.org).

Two-way analysis of variance (MedCalc version 8.1.1.0; MedCalc Software) was performed using the poly peptide amplitudes as dependent variable and time (0, 7, or 12 weeks) and diets (HFD or CD) as dependent factors. Amplitude distributions were tested to display signal alterations over time for different diets and to display significant interaction between both factors. Significance level was 0.05 for both tests. For correlation analysis (MedCalc version 8.1.1.0; MedCalc Software), Spearman’s rank correlation coefficients for normal data distributions were calculated.

Support Vector Machine Classifier Establishment—MosaCluster (version 1.7.0) (25) allows the classification of samples in the high dimensional parameter space by using support vector machines (SVMs). MosaCluster generates poly peptide classifiers, which rely on poly peptides displaying statistically significant differences, when comparing data from subjects with a specific disease to controls or other diseases. Each of these poly peptides represents one dimension in an n-dimensional parameter space (26–28). SVMs view a data point (subject’s urine sample) as an n-dimensional vector (n number of proteins used in the pattern), and they attempt to separate them with a (n – 1)-dimensional hyperplane. Of all possible hyperplanes, the one with the maximal margin to the nearest data points is selected. Classification is performed by determining the Euclidian distance (defined as the SVM score) of the n-dimensional vector to the (n – 1)-dimensional maximal margin hyperplane.

**Histology of Aortic Arches and Roots, Quantification of Plaque Size and Collagen Content**—After sacrificing the animals with an overdose of anesthetics, the chest was opened, and the aortic arch including the side branches carefully dissected. Aortic arches and aortic roots were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura, Torrance/CA, USA) and stored at −20 °C to obtain frozen sections. The arches were sliced longitudinally to their axis. Of each arch, at least 10 representative sections from the center of the vessel displaying the proximal branches were obtained and used for all further described histological analyses. Aortic roots were sliced horizontally, and at least 10 representative sections were evaluated and analyzed by histology. One subset of sections was stained using a standard Masson-Trichrome protocol. Plaque size was measured in at least three sections per animal from each group in mm2 using ImagePro Plus software (MediaCybernetics, Bethesda, MD). Furthermore, the collagen content of the plaque area was quantified using the same software. The total collagen content per plaque in mm2 and the collagen content as a percentage of the total plaque area were calculated.

**Immunohistochemistry of α1-Antitrypsin, Epidermal Growth Factor, and Collagen Type I**—To elucidate the potential role of α1-antitrypsin (α1-AT), epidermal growth factor (EGF), and collagen type I, immunohistochemistry was performed using the following antibodies: anti-α1-AT (Serotec, Oxford, UK), anti-EGF (BioWorld Technology, Minneapolis, MN), and anti-collagen type I (Millipore, Schwalbach, Germany). For antibody detection, we used an alkaline phosphatase detection kit (Vector Laboratories, Burlingame, CA). Staining intensity was quantified identically for the arteries and all side branches (0 = no stain; 1 = weak stain; 2 = medium stain; 3 = strong stain). Furthermore, plaque size in the immunohistology sections was evaluated in a semiquantitative manner in the aortic arch and all three side branches using an additive scoring system for each vessel (0 = no plaque; 1 = small plaque; 2 = medium plaques; 3 = large plaques), which was performed in all animals and averaged for each diet/treatment group. To confirm that expression of these marker proteins is not a general effect caused by a high fat diet, we also stained tissues from different organs (kidney, heart, skeletal muscle, lymph node, spleen, and liver), which were processed in the same manner as described in the previous paragraph.

**Immunohistochemistry of Human Plaques for α1-Antitrypsin, EGF, and Collagen Type I**—Atherosclerotic plaque samples were collected from routinely performed carotid endarterectomies. Sampling was approved by the Alfred Hospital Ethics Committee. Plaques were snap frozen in optimal cutting temperature compound and sectioned using a cryostat (Zeiss MICROM HM 550). Frozen sections were thawed at room temperature for 30 min and then fixed in acetone at −20 °C for 20 min. Subsequently, the sections were treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then incubated with normal serum for 30 min, followed by avidin and biotin blocking according to manufacturer’s recommendations (Vector Laboratories). After the blocking steps, the sections were incubated with primary antibodies overnight at 4 °C. The primary antibodies used were polyclonal rabbit anti-α1-antitrypsin (1:100 dilution; Biozol), polyclonal rabbit anti-collagen1 A1 (1:100 dilution; Biozol), and polyclonal rabbit anti-epidermal growth factor (1:50 dilution; Biozol). Detection was achieved by Vectastain ABC kit and diaminobenzidine substrate (Vector Laboratories).
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RESULTS

Identification of Mouse Urinary Biomarkers Reflecting Atherosclerotic Disease—Feeding ApoE<sup>−/−</sup> mice with a high fat diet represents a well accepted and highly consistent animal model of atherosclerosis. A systematic approach was undertaken to discover polypeptides that are specific for atherosclerosis. As a first step, we compared urine samples of ApoE<sup>−/−</sup> mice consuming HFD for 7 and 12 weeks (n = 20) versus urine samples from ApoE<sup>−/−</sup> consuming CD for the same time periods (n = 24; Table I). Appropriate statistical procedures considering multiple testing corrections could define 457 polypeptides of 1277, which could discriminate these two groups. In a second step, we compared urine samples of ApoE<sup>−/−</sup> mice consuming HFD for 7 and 12 weeks (n = 20) versus ApoE<sup>−/−</sup> at week 0 just before the start of their specific diet (n = 20). Herein, 469 urinary polypeptides could be defined as discriminators. In a third approach, we focused on early forms of atherosclerosis and compared urine samples from week 7 either HFD (n = 11) or CD (n = 12) with each other, resulting in 406 polypeptide discriminators. Comparing the three groups of polypeptides with each other, we could identify 106 polypeptides that are discriminative in all three comparisons.

HFD on its own is expected to cause changes in the urinary proteome. To exclude polypeptides that are not associated with atherosclerosis, we compared wild type mice before and after 12 weeks on HFD. We confirmed that these mice did not have atherosclerotic changes in the aorta and its associated arteries. Of the 106 previously defined discriminating polypeptides, 69 polypeptides were significantly altered by the HFD and were thus excluded from further investigation, resulting in 37 remaining polypeptides associated with atherosclerosis in mice.

To affirm the consistency of analysis, comparisons of the groups after randomization but before the start of specific diet of therapy were performed. Data analyses (n = 10 each group) did not identify a single polypeptide in urine that qualified as discriminator. This is a strong indication of the quality of analysis.

The identified 37 polypeptides were further analyzed using two-way analysis of variance to identify those candidates reflecting atherosclerosis. Therefore, amplitude distributions were tested to display signal alterations over time (0, 7, and 12 weeks) for different diets (HFD and CD, p < 0.05). Only candidates that revealed significant (p < 0.05) dependence on both diet and time with significant (p < 0.05) interaction between both factors were considered as polypeptide markers specific for atherosclerosis. Analysis of variance revealed 16 polypeptides of the 37 candidates fulfilling these criteria (Table II).

To identify the peptide sequence and post-translational modifications of these 16 potential biomarkers, tandem mass spectrometry was performed. Nine biomarkers could be identified, including fragments of collagen α1 (I), of major urinary proteins (3, 8, and 11), of uromodulin, of kidney androgen-regulated protein, of pro-epidermal growth factor, and of α<sub>1</sub>-antitrypsin (Table II and supplemental Figs. 1–9).

The 16 discovered biomarkers were used as an atherosclerosis-specific polypeptide classifier (Fig. 1) in SVM learning algorithms. A training set of ApoE<sup>−/−</sup> on HFD for 7 and 12 weeks (n = 20) versus ApoE<sup>−/−</sup> on CD for 0, 7, and 12 weeks (n = 34) was classified by a SVM model with an area under the ROC curve of 0.95 (95% CI 0.85–0.99) as evaluated by ROC analysis (supplemental Fig. 10A). A classification cutoff of −0.25 was selected; values above this threshold indicate significant atherosclerosis in comparison to no or minor background atherosclerosis (Fig. 2). Using this threshold, the training set was classified with a sensitivity of 95% (95% CI, 75–99) and specificity of 94% (95% CI, 80–99).

Validation of Mouse Urinary Biomarkers That Reflect Atherosclerotic Disease—The 16 identified biomarker candidates were evaluated in an independent test cohort consisting of 31
### TABLE II
Polypeptides constituting the arteriosclerotic progression specific polypeptide signature

Amp., mean amplitudes of atherosclerotic (ApoE<sup>−/−</sup> HFD, weeks 7 and 12) compared with control mice (ApoE<sup>−/−</sup> CD, weeks 0, 7, and 12); <i>ΔM</i>, mass deviation of theoretical peptide mass and analytical mass by CE-MS in parts per million; Weight, molecular weight (Da); ND, not detected; Regulation, ratio of mean amplitudes, positive values indicate up-regulation in atherosclerotic mice compared with controls; negative values indicate down-regulation in atherosclerotic mice compared with control.

| Peptide | Weight (Da) | CE time (min) | Amp. cases | Amp. controls | Regulation | Sequence | Parent protein | Residues | Accession number | M (ppm) | Best Mascot score | Observed m/z | Charge | Mascot score threshold |
|---------|-------------|---------------|------------|---------------|------------|----------|----------------|----------|-----------------|----------|-------------------|-------------|--------|---------------------|
| 4468    | 1,362.72    | 38.84         | 112        | 651           | −5.8       | SGNFDQTRVLN | Uromodulin     | 590–601  | Q91X17          | −23      | 42                | 682.3514   | 2      | 34                  |
| 5233    | 1,522.78    | 40.35         | 64         | 1304          | −20.3      | KpGEOGVpGDpGpGP | Collagen α-1(Ⅰ) chain | 646–661  | P11087          | −35      | 41                | 762.3711   | 2      | 37                  |
| 5321    | 1,539.79    | 40.16         | 2,188      | 104           | 21.1       | FLEQHLENSLV | Major urinary protein 1 | 30–42    | P11588          | 59       | 35                | 770.9223   | 2      | 33                  |
| 5725    | 1,638.84    | 40.17         | 2,273      | 355           | 6.4        | ND        | Collagen α-1(Ⅰ) chain | 530–549  | P11087          | −34      | 22                | 869.880    | 2      | 35                  |
| 6141    | 1,737.84    | 41.56         | 184        | 1056          | −5.7       | TGSqGSpGpGPp | Kidney androgen-regulated protein | 26–41    | P61110          | −4       | 84                | 900.9894   | 2      | 33                  |
| 6436    | 1,799.97    | 41.38         | 1,619      | 92            | 17.5       | SINKELQNSIIDLLNS | Kidney androgen-regulated protein | 25–41    | P61110          | −15      | 153               | 950.9230   | 2      | 33                  |
| 6564    | 1,826.96    | 35.42         | 950        | 90            | 10.5       | ND        | Kidney androgen-regulated protein | 25–41    | P61110          | −15      | 154               | 950.9230   | 2      | 33                  |
| 6843    | 1,899.06    | 41.88         | 13,018     | 932           | 14.0       | VSINKELQNSIIDLLNS | Kidney androgen-regulated protein | 25–41    | P61110          | −15      | 43                | 1,146.4955 | 2      | 27                  |
| 7189    | 1,998.13    | 42.24         | 11,115     | 1095          | 10.1       | ND        | Kidney androgen-regulated protein | 25–41    | P61110          | −15      | 154               | 950.9230   | 2      | 33                  |
| 7957    | 2,291.03    | 43.73         | 842        | 165           | 5.1        | EEISSNMRNFNEQI | Collagen α-1(Ⅰ) chain | 23–41    | P04393          | −22      | 31                | 1,146.4955 | 2      | 27                  |
| 8193    | 2,403.22    | 37.70         | 5,436      | 532           | 10.2       | WTDGMSPIREASLQGSDRLV | Pro-epidermal growth factor | 620–641  | P01132          | −14      | 53                | 1,202.9994 | 2      | 34                  |
| 8707    | 2,707.38    | 38.39         | 409        | 18            | 22.5       | ND        | α-1-Antitrypsin | 25–54    | Q00898          | 11       | 33                | 1,078.4956 | 3      | 30                  |
| 9236    | 3,232.43    | 41.96         | 820        | 82            | 10.0       | EVQETDTSQKDQSPASHEATNLGDFAI | α-1-Antitrypsin | 25–54    | Q00898          | 11       | 33                | 1,078.4956 | 3      | 30                  |

10459  | 4,523.21    | 30.68         | 896        | 12            | 74.7       | ND        | ND             | ND       | ND              | ND       | ND                | ND         | ND    | ND                  |
11004  | 5,940.97    | 30.78         | 658        | 90            | 7.3        | ND        | ND             | ND       | ND              | ND       | ND                | ND         | ND    | ND                  |
11347  | 9,219.24    | 37.02         | 444        | 23            | 19.5       | ND        | ND             | ND       | ND              | ND       | ND                | ND         | ND    | ND                  |
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Fig. 1. Atherosclerosis-specific polypeptide pattern. CE-MS profiling of mouse urine resulted in the definition of 16 atherosclerosis-specific polypeptides. Amplitude distributions were tested to display signal alterations over time (0, 7, and 12 weeks wk) for either a HFD or CD. Wild type (WT) mice were included to exclude polypeptides that are not associated with atherosclerosis, which does not evolve in WT mice, but that reflect nutritional proteome alterations caused by the HFD itself. Normalized molecular mass (800–15,000 Da) in logarithmic scale is plotted against normalized migration time (20–55 min). The mean signal intensity of the polypeptide peak is given in three-dimensional depiction.

samples from ApoE–/– mice comparing HFD week 7 (n = 10) and week 12 (n = 11) as with ApoE–/– mice before the start of diet (n = 10; Table I). Applying the established polypeptide classifier with the classification threshold of −0.25, 20 samples scored as atherosclerotic and 11 samples scored as no or minor atherosclerosis. This resulted in a sensitivity of 90% (95% CI, 70–99), in a specificity of 100% (95% CI, 69–100), and in an area under the ROC curve value of 0.97% (95% CI, 0.84–0.99) (supplemental Fig. 10B).

Atherosclerosis-specific Polypeptide Pattern Reflects Disease Progression—To test the hypothesis that the atherosclerosis-specific polypeptide patterns might constitute a sensitive tool to monitor atherosclerotic progression response in mice, we performed urine profiling to mice on HFD in addition to CD as control group at 0, 7, and 12 weeks (Table I and Fig. 2). ApoE–/– HFD and ApoE–/– CD at base line were not significantly different (p > 0.70), whereas ApoE–/– HFD displayed highly significant increase in mean scores after 7 (0.85 ± 0.64) and 12 (0.84 ± 1.01) weeks compared ApoE–/– CD (−1.21 ± 0.33 and −1.00 ± 0.58, p < 0.0001 for both comparisons).

Plaque Size, Collagen Content, and Immunohistochemistry (α1-AT, EGF, and Collagen Type I)—To further examine the reflection of the proteome pattern in plaque biology and the extent of atherosclerosis, we performed histological studies of atherosclerotic plaques, determined the extent of atherosclerosis, and screened for potential biomarkers such as collagen type I, α1-antitrypsin (α1-AT), and EGF.

Fig. 3 demonstrates Masson Trichrome stains of aortic arches and roots exemplarily comparing plaque size on ApoE–/– mice either on HFD or CD for 12 weeks. Large atherosclerotic plaques are seen in ApoE–/– mice on HFD (Fig. 3A), and the plaques are small in animals on a chow diet (Fig. 3B). Quantification of plaque size demonstrated that animals on HFD have a significantly higher plaque burden in aortic roots and arches compared with animals on CD (Fig. 3C).

Immunohistochemistry for collagen type I showed expression throughout the plaque area as well as in the adventitia of the vessel (Fig. 4, A and B). However, the extent of collagen expression in the percentage of total plaque area is similar for both groups (Fig. 4C).
The presence of $\alpha_1$-AT and EGF, of which degradation products were identified in urine, was also confirmed in the atherosclerotic plaques of mice (Fig. 5). Quantification of expression in atherosclerotic plaques demonstrated that ApoE$^{-/-}$ mice with HFD have significantly higher $\alpha_1$-AT expression levels in comparison with animals on chow diet (Fig. 5A). EGF expression was higher in animals on HFD in comparison with mice on chow diet (Fig. 5B).

The staining of other organs, such as kidney, heart, skeletal muscle, lymph node, spleen, and liver, showed no differences in the expression of antitrypsin, collagen, or EGF when comparing wild type mice with ApoE$^{-/-}$ mice on a high cholesterol diet (supplemental Figs. 11–13). This further confirms that the detected differences are specific for the atherosclerotic process and not a general phenomenon caused by differences in diet.

Correlation of Urinary Excretion Levels of Biomarkers with Histological Staining Intensity of the Respective Parental Proteins—Staining intensity of $\alpha_1$-AT in the mouse atherosclerotic plaques strongly correlated with the expression level of the respective $\alpha_1$-AT fragment detected in urine (Fig. 6A; $p < 0.01$). Also the amount of intraplaque collagen content, as quantified by Masson Trichrome staining, revealed a significant correlation with urinary excretion rates of the two identified collagen fragments (Fig. 6, B and C; $p < 0.02$ for both).

Evidence of Biological Significance of the Discovered Biomarkers in Human Atherosclerosis—The murine collagen fragment $\alpha_1$ (l) 530–549 with the amino acid sequence TGSpGSpGPDGKTGPpGPAG discovered here has also been identified by us in a recently published study on humans (29) with identical sequence and post-translational modifications. To provide further evidence for the applicability of our described proteomic discovery approach for human atherosclerosis, we performed immunohistochemistry of human atherosclerotic plaques for $\alpha_1$-AT, EGF, and collagen type I and could show a significant tissue expression of all these peptides in human atherosclerotic plaques (Fig. 7A). Furthermore, we were able to show strong differences by immunohistochemistry for these markers comparing areas with advanced atherosclerotic disease to those with only minor disease (Fig. 7, A versus B).
DISCUSSION

In this study, we describe a proteomic approach for the discovery and validation of murine biomarkers of atherosclerotic disease. In addition, we were able to apply this proteomic approach toward the discovery of proteins with a potential role in the pathophysiology of murine, as well as human atherosclerosis. Sequencing of the detected polypeptide biomarkers identified fragments of α1-antitrypsin, EGF, kidney androgen-regulated protein, and collagen type I, thereby describing these proteins as potential mediators and therapeutic targets in atherogenesis.

This study also indicates the potential of CE-MS-based urine proteome analysis to serve as a diagnostic tool for early detection and for monitoring of disease progression. The diagnostic potential of CE-MS has been shown previously for other diseases, such as urological disorders (30), chronic kidney disease (31), and graft versus host disease (32).

In a study recently published by our group, we were able to establish a proteome biomarker profile with the potential to noninvasively detect coronary artery disease in symptomatic patients with an accuracy of 84% (11). Among the identified novel urinary biomarker candidates were different collagen fragments, but none of the classical markers of plaque inflammation. The biomarker profile allowed the accurate diagnosis of coronary artery disease, which was superior to the sensitivity/specificity of other available noninvasive tests (3).

In the study described here, we were able to discover novel urinary biomarker candidates indicating early atherosclerosis stages in mice. Because early asymptomatic atherosclerotic disease cannot be reliably detected and quantified in humans, we used the well established ApoE−/− animal model in this study. As in previous studies, we focused on urine for analysis, giving its superior stability compared with plasma (11). Although in the study with symptomatic coronary artery disease patients all of the biomarkers identified were collagen fragments, in the current animal model we were able to identify additional new urinary biomarkers, which allowed diagnosis of significant atherosclerosis with a sensitivity of 90% and specificity of 100%.

Our study design offers a strong strategy of biomarker validation, because an independent validation cohort and a homogeneous reference standard in discovery and validation cohort were utilized. Furthermore, candidates identified by MS/MS peptide sequencing were validated in immunohistochemistry of relevant vessels and tissues. Finally, some of the rodent markers were detected in human samples using the identical CE-MS platform. This employed concept is in line with current guidelines for clinical proteome analysis (33, 34).

We could identify fragments of α1-AT as one of the significantly increased polypeptides in urine of atherosclerotic mice. Most importantly, α1-AT was also detectable in murine and human atherosclerotic plaques, and the expression level of α1-AT in the aorta of mice correlates with the extent of atherosclerosis and with the extent of its urinary excretion. The more α1-AT was detected in histology, the higher was the urinary excretion of the α1-AT fragment. These data indicate the potential of urine proteome analysis to directly reflect pathophysiological changes at atherosclerotic plaques. Several potential roles of α1-AT in atherosclerosis have been discussed previously: α1-AT can transform intraplaque monocytes into a pro-inflammatory state via interaction with scavenger and low density lipoprotein receptors and seems to induce oxidative stress (35–38).

Our data show a negative correlation between collagen plaque content and the quantity of collagen fragments found in urine, possibly caused by a decrease in collagen degra-
tion in more stable plaques, which would fit well to the reported findings of reduced metalloproteinase activity (39). Another protein identified in this study via urinary peptide sequencing, EGF, has been described in atherogenesis and plaque development, mediating monocyte chemotaxis, and macrophage proliferation (40, 41). The significantly increased expression of EGF in animals with HFD fits well with these observations, because enhanced monocyte/macrophage infiltration has been described in plaques of animals that consumed a Western-type diet (42).

To confirm that the expression of $\alpha_1$-AT, EGF, and collagen is not a general effect of the high fat diet, but specific for atherosclerosis, we have stained tissues from different organs (kidney, heart, skeletal muscle, lymph node, spleen, and liver) and found no difference in the expression of these three biomarkers in wild type mice compared with ApoE$^{-/-}$ mice. These data confirm the specificity of the detected changes in urine peptide patterns for atherosclerosis. It is especially important that we observed no significant protein expression differences in the kidneys of atherosclerotic and nonatherosclerotic mice, confirming that the differences in these biomarkers are caused “upstream,” before renal filtration and not by diet/atherosclerosis-related kidney alteration.

We could identify further proteins in the urinary proteome of atherosclerotic mice, such as kidney androgen-regulated protein, uromodulin, and major urinary protein. These proteins have not been described before to be directly associated with atherosclerosis. However, they have been found to be linked with other diseases, which are related to atherosclerosis. Kidney androgen-regulated protein seems to play a role in hypertension and renal alterations mediated by oxidative stress (43). Major urinary protein is involved in metabolic dysregulation in diabetic mice (44), and uromodulin (also called Tamm-Horsfall protein) is a urinary inhibitor of calcification (45). Overall, further detailed studies are warranted to elucidate the role of these proteins in atherosclerosis.

The study presented here was performed in a genetically homogenous group of ApoE$^{-/-}$ mice, which allowed biomarker discovery under controlled conditions. However, our results show impressive parallels to findings in humans (36, 46). Furthermore, our finding of the collagen $\alpha_1$-I fragment TGSpGSpGPDGKTGPpGPAG in the urine of atherosclerotic
mice and in patients with atherosclerotic disease (29), as well as our findings of the expression of \( \alpha_1 \)-AT, EGF, and collagen type I in human plaques, confirm the relevance of our animal study for the pathophysiology of human atherosclerosis. Furthermore, we could show a significant up-regulation of \( \alpha_1 \)-AT, EGF, and collagen type I when comparing areas with advanced atherosclerotic disease with those with only minor disease. Large scale studies are needed to confirm the diagnostic potential of these novel urinary biomarkers for early diagnosis, monitoring of progression, and therapy in humans.

The described urine peptide pattern is developed based on comparisons between mice with a high degree of atherosclerosis against mice with no or minor background atherosclerosis. These comparisons very realistically represent the clinical setting, for which a urine proteomic diagnostic test would be needed. Such a test should be suitable to discriminate patients that have higher degrees of atherosclerosis from those who have no or a minor degree of background atherosclerosis; the latter is to be expected in a large percentage of the population that would undergo a urine proteome testing.

A major advantage appears to be the concept of basing assessment not only on individual biomarkers, but also on the combination of a set of peptides. In addition, different peptides from the same parent protein provide a different discrim-
inatory potential, probably reflecting different protease activities that are characteristic of specific diseases. The approach goes beyond the specificity of individual biomarkers, which limits the potential of the classical single marker approaches.

The limitations of using individual biomarkers in atherosclerotic disease and its complications has been seen and the need to apply multimarker approaches has been generally acknowledged (47, 48). Our approach is following this line of biomarker development and goes a step beyond in that it is tailored to directly detect diagnostic patterns instead of individual diagnostic makers. Interestingly, recent evaluations of multimarker approaches that combine markers, which have been identified as individual biomarkers, to a multimarker assay have only demonstrated modest diagnostic improvements (47, 48). Overall, our approach is an example of a new strategy for biomarker discovery aiming toward pattern recognition.

Limitations—Although using state-of-the-art tandem mass spectrometry and despite major efforts, we were not able to sequence all 16 biomarker candidates. However, we may have reached the technical limits of currently available technology in top-down sequencing of naturally occurring peptides (49). In general, native peptide sequencing is limited by post-translational modifications, complicating not only peptide fragmentation, but also subsequent database searches (49, 50). In addition, the applied CE-MS technology is able to identify polypeptides with a high analytical sensitivity (17, 51), whereas tandem mass spectrometry used for sequencing has higher detection limits (52, 53).

To allow the assessment of the diagnostic potential of the described urine polypeptide pattern, an artificial yes/no-criteria for atherosclerosis was introduced, discriminating a high degree of atherosclerosis from no or a small degree of atherosclerosis. Because atherosclerosis is a chronically progressive disease, a quantitative measure representing the various degrees of atherosclerosis would be better suited for diagnostic purposes. This can be addressed in further extensive studies on the urine proteome that will include changes in abundance of the individual peptide fragments in relation to the extent of atherosclerosis.

Conclusions—A unique urine proteomic approach was used for the discovery and validation of novel urinary polypeptides that reflect atherosclerosis and its progression in ApoE−/− mice. Sequencing of these polypeptides identified fragments of α1-antitrypsin, EGF, collagen type I, and kidney androgen-regulated protein. The direct correlation of these urinary protein fragments with the histological evaluation of atherosclerotic plaques indicates the biological relevance of the identified proteins in atherogenesis. EGF, α1-antitrypsin, and collagen type I were also detected in human atherosclerotic plaques, demonstrating the potential of this approach to identify proteins relevant in human atherosclerosis. Overall, these data describe urine proteome analysis in an atherosclerotic mouse model as a discovery tool for novel pathogenic factors and therapeutic targets in human and mouse atherogenesis. These findings encourage further studies toward the development and application of atherosclerosis-specific proteome patterns for the noninvasive diagnosis of atherosclerosis in humans.

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