Quantitative Proteome Analysis Reveals Increased Content of Basement Membrane Proteins in Arteries From Patients With Type 2 Diabetes Mellitus and Lower Levels Among Metformin Users

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Background—The increased risk of cardiovascular diseases in type 2 diabetes mellitus has been extensively documented, but the origins of the association remain largely unknown. We sought to determine changes in protein expressions in arterial tissue from patients with type 2 diabetes mellitus and moreover hypothesized that metformin intake influences the protein composition.

Methods and Results—We analyzed nonatherosclerotic repair arteries gathered at coronary bypass operations from 30 patients with type 2 diabetes mellitus and from 30 age- and sex-matched nondiabetic individuals. Quantitative proteome analysis was performed by isobaric tag for relative and absolute quantitation-labeling and liquid chromatography–mass spectrometry, tandem mass spectrometry analysis on individual arterial samples. The amounts of the basement membrane components, α1-type IV collagen and α2-type IV collagen, γ1-laminin and β2-laminin, were significantly increased in patients with diabetes mellitus. Moreover, the expressions of basement membrane components and other vascular proteins were significantly lower among metformin users when compared with nonusers. Patients treated with or without metformin had similar levels of hemoglobin A1c, cholesterol, and blood pressure. In addition, quantitative histomorphometry showed increased area fractions of collagen-stainable material in tunica intima and media among patients with diabetes mellitus.

Conclusions—The distinct accumulation of arterial basement membrane proteins in type 2 diabetes mellitus discloses a similarity between the diabetic macroangiopathy and microangiopathy and suggests a molecular explanation behind the alterations in vascular remodeling, biomechanical properties, and aneurysm formation described in diabetes mellitus. The lower amounts of basement membrane components in metformin-treated individuals are compatible with the hypothesis of direct beneficial drug effects on the matrix composition in the vasculature. (Circ Cardiovasc Genet. 2015;8:727-735. DOI: 10.1161/CIRCGENETICS.115.001165.)

Key Words: cardiovascular diseases ■ diabetes mellitus, type 2 ■ extracellular matrix ■ metformin ■ muscle, smooth

The association of type 2 diabetes mellitus (T2DM) with an increased risk of several cardiovascular diseases (CVDs) has been extensively documented, but the origins of the association remain largely unknown. Several studies have shown that an increased risk of CVD in DM occurs independently of the presence of hypercholesterolemia, hypertension, and other well-known risk factors, but that CVD in DM is associated with the presence of microvascular complications.

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The arterial pathology in DM does not only include increased occurrence of atherosclerotic plaques but also comprises generalized arterial alterations, that is, endothelial dysfunction, increased stiffness, extracellular matrix (ECM) changes and calcifications, which can be observed in both atherosclerotic and nonatherosclerotic parts of the arterial tree. Generalized changes in the vascular ECM have been described among patients with T1DM, that is, for glycosaminoglycans, glycoproteins, and collagens. More recently, alterations in the levels of the metalloproteinase
2, an elastin cleaving protease, were observed in human arteries from patients with T2DM.\(^7\)\(^8\) Moreover, in a study based on RNA-microarray examinations of nonatherosclerotic arterial tissue from T2DM patients, we found altered expression of a matrix-related pathway\(^9\) and increased expression of the basement membrane protein fibulin-1, both at the mRNA and protein level, in arterial samples from patients with T2DM.\(^10\) Alterations in the arterial matrix could be involved in the increased arterial stiffness,\(^11\) in the dysfunctional remodeling (shrinkage instead of compensatory enlargement of damaged arteries),\(^12\)\(^13\) and in the paradoxically decreased risk of abdominal aortic aneurysms described among patients with T2DM.\(^14\)\(^15\) Likewise, altered arterial matrix influence atherosclerosis and alter responses to injury.\(^17\)

Worldwide, the most prescribed antidiabetic drug is metformin, shown to display beneficial cardiovascular effects in patients with T2DM.\(^16\) It exerts blood glucose–lowering effects mainly through the inhibition of the gluconeogenesis in the liver. The primary cellular effect of metformin has been ascribed to its ability to activate the AMP kinase.\(^19\) A recent article suggests that the precise molecular explanation is based on drug effects on the mitochondrial glycerophosphate dehydrogenase.\(^20\)

Metformin exerts beneficial cardiovascular effects in patients with T2DM by improving endothelial function\(^21\) and decreasing the level of circulating endothelial markers.\(^22\) In addition, we have, in a randomized study, shown that plasma levels of the basement membrane protein fibulin-1 are reduced by metformin, unrelated to the glycemic control.\(^23\) In relation to the development of diabetic complications, it is also of interest that a study recently showed that mitochondrial dysfunction and reduced activated protein kinase (AMPK) activity in DM are involved in the development of matrix accumulation and proteinuria as part of the diabetic kidney complications and that the kidney damage was corrected by another AMPK stimulator, that is, 5-Aminoimidazole-4-carboxamide ribonucleotide.\(^24\) Thus, it can be hypothesized that metformin through AMPK activation may exert beneficial effects on matrix accumulation as part of diabetic vascular complications.

In this study, we aimed at obtaining knowledge about the molecular pathology of the diabetic arteriopathy by comparing nonatherosclerotic tissue from the internal mammary arteries from patients with T2DM with tissue from patients without DM by quantitative proteomics, focusing particularly on matrix proteins. Moreover, we test whether the use of metformin among patients with DM is associated with altered expression of proteins in arterial tissue. The internal mammary artery has proven to be a suitable model artery for investigations of generalized nonatherosclerotic arterial changes because, for example, its matrix composition and endothelial function and biochemistry reflect alterations in both the coronary and carotid arteries and other vessels.\(^10\)\(^25\)\(^26\)

**Methods**

**Patients and Arterial Material**

The repair artery from coronary bypass surgery (the internal mammary/the internal thoracic artery) was collected at the Department of Thoracic, Heart and Vascular Surgery, Odense University Hospital, consecutively from 2008 to 2012. Arterial tissue from 35 patients with T2DM was collected from our tissue collection, together with arteries from a group of 35 age- and sex-matched nondiabetic individuals. Individuals were assigned as having T2DM diagnosis, if this diagnosis was registered in the patients file or if at least one hemoglobin A1c (HbA1c) measurement was >6.5%. Patients were found negative for GAD antibodies in a blood sample taken the day before the operation. Nondiabetics had HbA1c measurement <6.0%. All of the patients (both diabetic and nondiabetic) received statins. A blood sample was taken for lipid values, and the patient was questioned about current and previous smoking habits. Information about medications, body weight, and height was extracted from the medical files. All participants gave written informed consent, and the study was approved by the local ethics committees (S-20100044).

Immediately after surgery, the internal mammary artery was dissected free from the surrounding tissue, that is, muscle, fat, and the outer part of adventitia by a person unaware of the DM status of the patient. An arterial ring was cut and short-term formalin-fixed (≈24 hours in 4% buffered paraformaldehyde), then moved to a PBS solution and subsequently embedded in paraffin. The paraffin blocks were cut into sections of 4 μm and elastin stained, and all samples had <5% of the circumference covered with intima lesions. Nevertheless, 5 patient samples (and matching individuals) had to be removed because of minute amounts of tissue or incorrect embedding. The final group for proteome analysis therefore consists of 30 diabetic and 30 nondiabetic individuals (Table). A detailed description of the histology, quantitative histomorphometry, and immunohistochemistry is given in the Data Supplement.

**Proteome Analysis of Paraffin-Embedded Formalin-Fixed Artery Tissue**

**Extraction and Digestion of Proteins in Spinfilters**

Our proteome analysis was based on material from 1 slice of the same formalin-fixed material as used for histology using a filter-aided digestion protocol modified from the study of Wiśniewski et al.\(^2\) The paraffin was dissolved by washing with chloroform, and the tissue was released from the object glass and transferred to an Eppendorf tube in 2-μL extraction buffer (40 mmol/L triethyl ammonium bicarbonate pH 8, 4% SDS, 100 mmol/L dithiothreitol). The samples were incubated 20 minutes at 99°C followed by 120 minutes incubation at 80°C with 600 rpm agitation. The extracted proteins were added 2× 200 μL 8 mol/L urea in 40 mmol/L triethyl ammonium bicarbonate pH 8 and centrifuged through a 3000 molecular weight cut-off spin filter (Millipore Amicon Ultra) at 14000 g at room temperature. Proteins were alkylated in the spin filter using 50 mmol/L iodoacetamide in 8 mol/L urea for 30 minutes in the dark. The buffers were exchanged by spinning 2× 100 μL 8 mol/L urea and 2× 100 μL 200 mmol/L triethyl ammonium bicarbonate through the filters before digesting overnight with 1 μg trypsin (Promega) at 37°C.

**8-Plex iTRAQ Labeling**

Based on a nanodrop protein measurement, 3.5 μg peptides from each patient were labeled with isotopic tag for relative and absolute quantitation (iTRAQ) 8-plex reagent according to the manufacturer’s description (Applied Biosystems, CA). In a series of investigations, the samples were labeled as follows—Label 113: pool non-DM, 114: pool DM, 115: non-DM 1, 116: DM 1, 117: non-DM 2, 118: DM 2, 119: non-DM 3, and 121: DM 3 (non-DM 1 and DM 1+non-DM 2 and DM 2+non-DM 3 and DM 3 are matched patient pairs). The labeled peptides were mixed in equal amounts into a total of ten 8-plex samples. Liquid chromatography–mass spectrometry, tandem mass spectrometry analyses were performed on these mixed samples with 6 individual samples (and 2 pools) per run. A detailed table describing the analytic design can be found in the Data Supplement (Table I in the Data Supplement).

**Reversed Phase Desalting of Peptides and Offline HILIC Fractionation**

The acidified samples were desalted using a reversed phase microcolumn as used for histology using a filter-aided digestion protocol and subsequently embedded in paraffin. The paraffin blocks were cut into sections of 4 μm and elastin stained, and all samples had <5% of the circumference covered with intima lesions. Nevertheless, 5 patient samples (and matching individuals) had to be removed because of minute amounts of tissue or incorrect embedding. The final group for proteome analysis therefore consists of 30 diabetic and 30 nondiabetic individuals (Table). A detailed description of the histology, quantitative histomorphometry, and immunohistochemistry is given in the Data Supplement.
**Online Reversed Phase LC Separation and Mass Spectrometric Analyses**

The mass spectrometric analyses of the HILIC fractions were performed on a Q-Exactive (Thermo Scientific) instrument coupled to a Dionex UltiMate 3000 nano high-performance liquid chromatography using a 42-minute linear gradient and a TSKgel amide-80 HILIC column.

### Data Processing and Quantification

All Q-Exacte raw data files were processed and quantified using Proteome Discoverer version 1.4.0.288 (Thermo Scientific). The Mascot search Engine v. 2.2.3 integrated with Proteome Discoverer was used to search the data with the following criteria—protein database: Swissprot; enzyme: Trypsin/P; maximum missed cleavage sites: 2; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.1 Da; fixed modifications: carbamidomethyl (C), iTRAQ8plex (K), and variable modifications: oxidation (M).

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**Table. Clinical Data in the Patient Groups Undergoing Coronary Bypass Operations: Patients Without (Non-DM) and With T2DM, and Subdivision of the DM Patients Treated Either Without (T2DM–M) or With Metformin (T2DM+M)**

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Non-DM</th>
<th>T2DM</th>
<th>P Value</th>
<th>T2DM–M</th>
<th>T2DM+M</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>30</td>
<td>30</td>
<td></td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Age at surgery, y</td>
<td>68.2±1</td>
<td>67.9±0.8</td>
<td>0.865</td>
<td>67.6±1.1</td>
<td>68.2±1.3</td>
<td>0.717</td>
</tr>
<tr>
<td>DM duration, y</td>
<td>...</td>
<td>7.7±1.3</td>
<td>...</td>
<td>8.5±2.6</td>
<td>7±1.1</td>
<td>0.583</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>83</td>
<td>83</td>
<td></td>
<td>79</td>
<td>88</td>
<td>0.529</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.6±0.1</td>
<td>7.0±0.2</td>
<td>&lt;0.001</td>
<td>7.2±0.3</td>
<td>6.8±0.2</td>
<td>0.347</td>
</tr>
<tr>
<td>HbA1c, mmol/mol</td>
<td>38±1</td>
<td>53±2</td>
<td>&lt;0.001</td>
<td>55±3</td>
<td>51±2</td>
<td>0.347</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.3±0.2</td>
<td>3.8±0.2</td>
<td>0.115</td>
<td>4±0.3</td>
<td>3.6±0.2</td>
<td>0.279</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.4±0.2</td>
<td>1.9±0.1</td>
<td>0.025</td>
<td>2.1±0.2</td>
<td>1.7±0.1</td>
<td>0.116</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.4±0.1</td>
<td>1.1±0.05</td>
<td>0.011</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>0.766</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3±0.1</td>
<td>2.0±0.2</td>
<td>0.001</td>
<td>2.0±0.3</td>
<td>1.9±0.2</td>
<td>0.754</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>87±3.1</td>
<td>97±5.1</td>
<td>0.129</td>
<td>97±7.8</td>
<td>97±6.8</td>
<td>0.958</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27±0.7</td>
<td>29±0.8</td>
<td>0.019</td>
<td>29±1.3</td>
<td>30±1</td>
<td>0.632</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>139±3.5</td>
<td>140±3.3</td>
<td>0.804</td>
<td>138±3.1</td>
<td>142±5.7</td>
<td>0.553</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>77±1.9</td>
<td>74±4.2</td>
<td>0.382</td>
<td>74±3.4</td>
<td>75±2.3</td>
<td>0.879</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>13</td>
<td>15</td>
<td>0.612</td>
<td>6</td>
<td>9</td>
<td>0.481</td>
</tr>
<tr>
<td>Medication</td>
<td>Statins, n</td>
<td>30</td>
<td>30</td>
<td>...</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Antihypertensives (RAAS), n</td>
<td>15</td>
<td>25</td>
<td>0.013</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Other antihypertensives, n</td>
<td>26</td>
<td>28</td>
<td>0.398</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Antiplatelet agents, n</td>
<td>29</td>
<td>29</td>
<td>1</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Insulins, n</td>
<td>0</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>Antibiotic agents, n</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>16</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results are shown as mean±SEM, percentage (%), or number (n), as indicated. BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RAAS, renin-angiotensin-aldosterone system; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

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**Statistical Analysis**

Proteome results, as well as histomorphometric and clinical parameters, are expressed as mean±SEM or as number or percentage, when
Results

Patient characteristics for the patient groups with and without T2DM are presented in Table, where it is obvious that diabetic patients had increased HbA1c, higher body mass index, lower low-density lipoprotein and higher-density lipoprotein, and higher plasma triglyceride.

All 129 identified proteins are displayed in the Table in the Data Supplement, and as can be seen in Figure 1A and in Table II in the Data Supplement, the α-1 chain of type IV collagen (the most abundant collagen type of basement membranes) was increased by 41% in T2DM (P=0.00006 [t test], P=0.0074 with Benjamini–Hochberg multiple testing correction). Likewise, other basement membrane molecules, such as collagen α2-type IV, collagen α1-type XVIII, laminin-γ1, and laminin-β2 (Figure 1B–1E; Table II in the Data Supplement), were found to be increased in DM, when simple t tests were used for statistical analysis, although these differences did not reach significance levels when correction for multiple testing was taken into account. Noteworthy, however, when the probability of the observed distribution of proteins with t test calculated P values below or above 0.05 was compared between the group of identified basement membrane peptides (α1-collagen type IV and α2-collagen type IV, α1-collagen XVIII, laminin-γ1, laminin-β2, nidogen, and perlecan; 5 with P<0.05 of 7) and the group of all other proteins (21 with P<0.05 of 122), the P value was 0.0037 using a 2×2 table and Fisher’s exact test. Moreover, trends for the basement membrane components, nidogen-1, were seen as well (Figure 1F; Table II in the Data Supplement). The only identified basement membrane molecule, which did not display signs of a difference, was perlecan (basement membrane heparan sulfate proteoglycan; Figure 1G; Table II in the Data Supplement). No changes were observed for any interstitial collagens, that is, type I, III, V, and VI (Figure 1H; Table II in the Data Supplement), and likewise none of other identified glycoproteins or proteoglycans showed differences, except biglycan, which displayed higher concentrations in DM (Table II in the Data Supplement). A few abundant cellular proteins showed increased amounts in DM, as for example desmin (Figure 1I; Table II in the Data Supplement), whereas other cellular proteins, that is, vimentin and α-smooth muscle actin were unchanged (Figure 1J; Table II in the Data Supplement). We did not observe correlations between values of α1-type IV collagen and HbA1c or other clinical parameters, when analyzed in the 2 patient groups separately.

Several of the DM-related, differentially regulated proteins displayed lower levels in the patient subgroup treated with metformin, that is, the α-chains of type IV collagen, laminin-γ1, nidogen-1, and collagen type XVIII (Figure 1A–D and 1F; Table II in the Data Supplement). Some other upregulated proteins in DM were also changed in metformin-treated patients, that is, desmin, cytoplasmic actin-1, and myosin11 (Figure 1I; Table II in the Data Supplement). Noteworthy, the smooth muscle cell abundant glycolysis enzyme, phosphoglucomutase-like protein 5 (PGM5), was decreased in metformin users (Table II in the Data Supplement).

The quantitative histomorphometric analysis is summarized in Table III and Figure 1 in the Data Supplement (all images) and in Figure 2. The samples were without significant intimal atherosclerosis, and no macrophages or other cellular infiltrates were observed in any sample. The quantitative histomorphometric analysis showed that the area fraction of collagen-stainable material was significantly increased in the intima and borderline in the media layers (Figure 2; Table III in the Data Supplement), whereas no significant changes were seen when metformin- or nonmetformin-treated patients were compared in the DM group. No changes were observed in the area fraction of elastin-stainable material (Table III in the Data Supplement). Counting of nuclear profiles displayed that there were no statistically significant differences in the density of smooth muscle cell nuclear profiles in tunica intima and tunica media, but that the number of smooth muscle cells were significantly reduced in tunica adventitia in individuals with T2DM (Figure 2; Table III in the Data Supplement). Because of variations of the massive staining intensities of basement membrane components in the tunica media and variation both in individual arteries, as well as between samples, it was not possible to develop a useful quantitative immunohistochemistry protocol for basement membrane components. However, examples of type IV collagen and laminin immunostaining, performed in 3 patients from the non-DM group and 6 from the diabetic group (Figure 3), showed abundant pericellular amounts of basement membrane material throughout the tunica media (Figure 3A–3F).

Discussion

Our novel observation of increased content of type IV collagen and several other arterial basement membrane constituents in arterial tissue from individuals with T2DM is surprisingly distinct. Apart from biglycan, none of the other 20 quantified ECM proteins are altered in DM. Importantly, the investigated tissue is normal appearing without atherosclerosis and without macrophages or other cellular infiltrates, showing that the underlying explanation is not because of local inflammation, but may be related to effects of metabolic or hormonal factors, related to the diabetic milieu. Our quantitative histomorphometric investigations support the proteome data because we observed increased staining for Masson trichrome stainable material (collagen) in the tunica intima and close to significant changes in the tunica media. It should however be mentioned that additional validation of individual protein results is needed in arterial tissue from other DM populations. Importantly, we did not observe significant changes in cell number in the largest part of the studied vessels, that is, tunica media, although a tendency toward lower cell numbers was seen in DM and significant lower smooth muscle cell counts were found in a small area outside the external elastic membrane in tunica adventitia. Increased amounts of cells do therefore not seem to explain accumulation of the basement membrane components.
Interestingly, one structural hallmark of the diabetic microangiopathy is accumulation of basement membranes, which among patients with DM has been observed in small vessels in different places of the body, that is, in the glomeruli, muscle capillaries, and retinal vessels. Basement membrane accumulation has most often been observed by electron microscopy, whereas biochemical data are scant; however, our results are compatible with the idea that at least some of the mechanisms behind macrovascular and microvascular complications in DM may be common. In the arterial wall, the majority of the basement membrane material is situated around the vascular smooth muscle cells in tunica media, as previously reported, as observed in this study. The most abundant proteins in basement membrane areas are type IV collagen and laminin, but also nidogen, fibulin-1, type XVIII collagen, and basement membrane heparan sulfate proteoglycan (perlecan) are part of the protein constituency. In contrast to the other identified basement membrane molecules, we did not observe changes in the arterial concentration of basement membrane heparan sulfate proteoglycan, which fits well with a few older reports about measurements of perlecan in glomeruli, showing that the amount of this molecule is actually reduced in DM, when judged in relation with the amount of collagen. Importantly, although these data, also concerning laminin chains, may
indicate that not only the amount but perhaps also the qualitative composition of the arterial basement membranes is changed in DM, a conclusion will have to await further studies.

There are only few reports about the structural or molecular pathology of the arterial wall in T2DM, however, our observations fit well with a few other studies, which have reported altered amounts of the metalloproteinase matrix metalloproteinase 2\(^{27}\) and miR-221/222\(^{38}\) in normal appearing arteries from patients with T2DM. Moreover, the present observations are compatible with the previous findings of changes in ECM-related pathways in a transcriptomic investigation of arterial tissue from patients with T2DM,\(^9\) where we on the basis of a hypothesis-free transcriptomic analysis observed, that the basement membrane protein fibulin-1 is present in increased amounts both at the RNA, but also on the protein level in arterial tissue in DM. Interestingly, we subsequently found that fibulin-1 is a circulating marker for arterial stiffness and CVDs in T2DM and an independent predictor of mortality.\(^{10}\) Our present observations are compatible with these previous reports and raise the question whether alterations in basement membrane constituents are related to arterial dysfunctions and cardiovascular symptoms among patients with DM.

Pathophysiologcal consequences of alterations in arterial basement membranes are not extensively studied; however, some recent genetic and experimental data strongly indicate that basement membrane changes leads to functional and structural alterations of the arterial wall relevant to the diabetic arteriopathy. Induced DNA changes in mice and rare genetic conditions in humans have shown that mutations in both the \(\alpha\)-1 and \(\alpha\)-2 chains of type IV collagen, which disrupts the basement membrane collagen network, are followed by arterial fragility and intracerebral hemorrhage (porencephaly) and hereditary angiopathy, nephropathy, aneurysms and muscle cramps (HANAC).\(^{39}\) Dysfunction of arterial smooth muscle cells seems to explain these phenotypes in line with the observation that mice with col4a1 missense mutations display defects in several vascular functions and low blood pressure.\(^{40}\) In addition, it is interesting that a polymorphism in \(\alpha\)-1-collagen type IV in a genome-wide association study was found significantly associated with arterial stiffness.\(^{41}\) Thus, variations in \(\alpha\)-1 and \(\alpha\)-2 chains of type IV collagen regulate the capacity of arterial remodeling and determine stiffness, aneurysm formation, etc. This knowledge in combination with our observation of increased amounts of these collagen chains...
in T2DM therefore fits well with the novel idea that basement membrane accumulation may constitute at least part of the molecular background for increased arterial stiffness, dysfunctional remodeling, and increased protection against abdominal aneurysms, which prevails in DM.

Another striking observation in the present study is the reduced levels of many of the upregulated basement membrane proteins in the subgroup of type 2 diabetic patients treated with metformin. This effect is present despite the fact that all registered clinical parameters, including HbA1c levels, body mass index, and DM duration were similar in metformin-treated and not-treated patients. It is therefore possible that the observed differences are related to more direct effects of the drug, but not related to glycemic status, although we cannot exclude possible influences of factors related to the indication for the use of metformin or other antidiabetics. It should be noted that even though the protein expression of most basement membrane proteins are reduced in the metformin-treated group, the histomorphometric trichrome analysis is not. This may indicate that although collagen IV is reduced by metformin, total collagen is not to the same degree. A more detailed insight into the interaction between DM, metformin, and collagens will, however, have to await further studies. Nevertheless, the present results fit well to recent observations in a randomized study, where metformin reduced plasma concentrations of the basement membrane protein fibulin-1 independent of glycemia, strongly suggesting that the use of metformin exerts effects in pathways directly involved in vascular matrix production. This notion is moreover compatible with recent experimental results showing that AMP-kinase (activated by metformin) is reduced in kidney tissue in experimental DM and that activation of the enzyme (with AICAAR) normalizes matrix accumulation and albuminuria. Moreover, our findings fit with previous results of reduced activity of AMP-kinase in insulin resistance and endothelial dysfunction and also with data showing influences of AMP kinase activity on transforming growth factor- signaling and matrix accumulation. The putative role of AMP kinase in arterial matrix production is supported by the fact that AMP kinase–deficient mice are protected against the development of abdominal aneurysms, a condition emerging on the basis of dysfunctional matrix turnover, again fitting well with the present results and with the fact that patients with DM are protected against the development of abdominal aortic aneurysms.

Our study is based on individual quantitative proteome analysis on 60 well-characterized homogeneous samples collected from our artery biobank of internal mammary arteries from several hundred individuals. No standard protocols exist for proteome analysis of arterial material because only few studies of human vascular tissue have been done. The design of our proteome analysis was based on a demand to achieve quantitative data on an individual basis, to be able to estimate the biological variance in the patient groups. This was obtained by the use of iTRAQ labeling of individual samples and by the use of an internal standard in each analytic run (a pool of nondiabetic samples). This led to quantitative results for each protein in each sample and the possibility to do comparisons between groups with proper statistical tests also involving correction for multiple testing. Moreover, we needed to be able to use small amounts of well-defined tissue and to include as many ECM proteins as possible. These demands were obtained using sections of formalin-fixed, paraffin-embedded arterial samples combined with a simple protein extraction/degradation protocol compatible with the iTRAQ labeling procedure. The design of the present proteome analysis led to quantification of a modest number of identified proteins (128, Table II in the Data Supplement), but comparable with the number obtained in a few proteomic studies on arterial material. Our identification and quantification of several biochemically different proteins from the arterial wall is in line with previous investigations, showing that proteome analysis can be done effectively on formalin-fixed material. Proteome analysis of arterial tissue from patients with DM has not previously been performed; however, recently
this technique was used to 10 (2×5) aortas from streptozotocin-induced diabetic mice.50 There is not much overlap in findings between our study and the list of proteins with apparent alterations in the experimental investigation; however, the proteome protocol in the experimental study only identified few matrix molecules. Moreover, the effect of short-term streptozotocin-induced DM in mice leading to plasma glucose concentrations >20 mmol/L is probably not relevant when compared with the clinical situation prevailing in arteries from patients with long-term T2DM.

All our included patients had coronary atherosclerosis because they undergo a coronary bypass operation, and we cannot exclude that this selection may interfere in the interpretation of our results, however, comparable arteries from individuals without coronary atherosclerosis are unfortunately not obtainable. Likewise, it should be mentioned that the included patients with T2DM also have increased body mass index and lipid alterations, which makes it difficult to deduce if the observed effects are because of the presence of DM, obesity, lipid changes, or a combination.

In conclusion, we show that basement membrane proteins accumulate in nonatherosclerotic arteries from overweight patients with T2DM. Augmented amounts of basement membrane material in the arterial wall resemble one of the elements of small vessel disease in DM and seem to suggest that common disease pathways exist in the microangiopathy and the macroangiopathy in T2DM. The observed arterial ECM alterations are likely to constitute the basis for the development of at least some of the arterial manifestations seen in DM, that is, increased stiffness, dysfunctional remodeling, and protection against abdominal aortic aneurysms, and may also be involved in acceleration of some elements of the atherogenic process. Moreover, our data show that metformin intake is associated with less accumulation of basement membrane proteins, an observation compatible with the hypothesis that beneficial cardiovascular effects of metformin may be related to effects on the matrix composition of the arterial wall.

Sources of Funding
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Disclosures
None.

References
Moreover, our results point out that metformin effects may influence the arterial pathology in diabetes mellitus. Increased arterial stiffness, medial calcifications, and dysfunctional remodeling are elements of the generalized alterations present in the arterial wall in type 2 diabetes mellitus, which leads to augmented cardiovascular morbidity. These generalized arterial alterations may also be involved in the protection against aneurysms in diabetes mellitus. The knowledge about the molecular pathology of the arterial wall in diabetes mellitus is nevertheless very scant, and we have therefore performed proteome analysis on human arterial samples and find that the concentrations of arterial basement membrane proteins are distinctly upregulated in type 2 diabetes mellitus. Accumulation of basement membrane components is present in the glomerular and capillaries in the mouse brain. COL4A1 is associated with arterial stiffness by genome-wide association scan. Circ Cardiovasc Genet. 2009;2:151–158. doi:10.1161/CIRCGENETICS.108.823245.


Quantitative Proteome Analysis Reveals Increased Content of Basement Membrane Proteins in Arteries From Patients With Type 2 Diabetes Mellitus and Lower Levels Among Metformin Users

Simone A.R. Preil, Lars P. Kristensen, Hans C. Beck, Pia S. Jensen, Patricia S. Nielsen, Torben Steiniche, Marina Bjørling-Poulsen, Martin R. Larsen, Maria L. Hansen and Lars M. Rasmussen

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SUPPLEMENTAL MATERIALS

Supplemental Methods. Histomorphometry and immunohistochemistry

Paraffin sections from all arterial samples were cut in 4 µm sections and stained by routine methods for hematoxyline and eosin (HE), elastin (Weigert), collagen (Masson trichrome) and by immunohistochemistry for macrophages (CD68, Dako KP-1 (1:800) and Dako PG-M1 (1:100)) and smooth muscle cells (SMC α-actin, Dako 1A4 (1:50)) using UltraView Universal Alkaline Phosphatase Red Detection Kit from Ventana Medical Systems and for type IV collagen (Cell Marque, clone CIV22) and laminin (Dako, clone 4C7) using UltraView Universal Horse Radish Peroxidase (HRP) Detection Kit from Ventana Medical Systems. Control sections included sections of carotid atherectomies, which showed areas positive for macrophages and smooth muscle cells and sections incubated without primary antibodies, which were negative. To determinate the area fractions of collagen and elastin stainable material, as well as the numbers of smooth muscle cells, in the arterial samples, the three arterial layers were outlined by a person, unaware of patient characteristics: The luminal border of the vessel could be determined by the analyzing software itself, however the border between intima and media was drawn manually along the internal elastic membrane, and the border between tunica media and tunica adventitia was drawn along the external elastic membrane. Subsequently, the adventitial area was defined by a line 30 µm parallel abluminal to the line defining the external elastic membrane. This procedure defined three areas, i.e. intima, media and adventitia and quantitative analysis of the area fraction of stainable material was done using Visiopharm Integrator System 4.2.3.0 (Visiopharm A/S, Hørsholm, Denmark). Using this software, nuclear profiles (stained blue) of smooth muscle cells (cytoplasm stained red) and non-smooth muscle cells were counted too.
Supplemental Table S1. Design of proteome analysis.

This table displays how different samples (T2DM: D1-D30 and non-DM: C1-C30) were labelled with the 8-plex iTRAQ-reagents (113, 114, 115, 116, 117, 118, 119 and 121) and how these labelled samples were examined in 10 different LC-MS/MS analytical runs (Run# A-J), as described in the method section. As can be seen a pool of both non-DM samples (C1-C30) and T2DM samples (D1-D30) were included in each run. Results of the individual runs are displayed as the ratio of the individual label divided with iTRAQ-113 (non-DM pool, used to normalize between all runs) in the individual runs. The ratio between label 114 and 113 for each run gives rise to the determination of analytical precision. All results can be found in the attached excel-sheets (Table S2, A, B, C, D).

<table>
<thead>
<tr>
<th>Run #</th>
<th>iTRAQ-label</th>
<th>113</th>
<th>114</th>
<th>115</th>
<th>116</th>
<th>117</th>
<th>118</th>
<th>119</th>
<th>121</th>
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<tr>
<td>Search file nomenclature</td>
<td>Excell-file nomenclature</td>
<td>Sample numbers:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AMix1</td>
<td>A5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C1</td>
<td>D1</td>
<td>C2</td>
<td>D2</td>
<td>C3</td>
<td>D3</td>
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<tr>
<td>BMix2</td>
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<td>DM-pool</td>
<td>C8</td>
<td>D8</td>
<td>C4</td>
<td>D4</td>
<td>C5</td>
<td>D5</td>
</tr>
<tr>
<td>CMix3</td>
<td>C5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C6</td>
<td>D6</td>
<td>C7</td>
<td>D7</td>
<td>C30</td>
<td>D30</td>
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<td>D5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C9</td>
<td>D9</td>
<td>C10</td>
<td>D10</td>
<td>C11</td>
<td>D11</td>
</tr>
<tr>
<td>EMix5</td>
<td>E5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C12</td>
<td>D12</td>
<td>C13</td>
<td>D13</td>
<td>C14</td>
<td>D14</td>
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<tr>
<td>FMix6</td>
<td>F5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C15</td>
<td>D15</td>
<td>C16</td>
<td>D16</td>
<td>C17</td>
<td>D17</td>
</tr>
<tr>
<td>GMix7</td>
<td>G5</td>
<td>nDM-pool</td>
<td>DM-pool</td>
<td>C18</td>
<td>D18</td>
<td>C19</td>
<td>D19</td>
<td>C20</td>
<td>D20</td>
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<tr>
<td>HMix8</td>
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<td>DM-pool</td>
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<td>D22</td>
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<td>DM-pool</td>
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<td>D25</td>
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<td>C28</td>
<td>D28</td>
<td>C29</td>
<td>D29</td>
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</table>
Supplemental Table S2. Proteome results: raw data, MASCOT scores, normalized values and analytical precision.

Data are displayed in accompanying excel file (sheet A, B, C and D).

A. (Raw data). The expression of all identified proteins including peptide and protein data. Nomenclature in row 1 refers to the run and iTRAQ-label, as explained in Supplemental Table S1.

B. (FINAL LIST uncorrected). The expression of 129 identified and quantitated proteins, present in at least eight out of 10 runs (at least 48 individuals) is shown. Results from diabetic (D1-D30) and non-diabetic (C1-C30) (row 1) samples are displayed together with LC-MS/MS run number and iTRAQ-label (row 2), as explained in Supplemental Table S1. Metformin treated patients are shown in yellow.

C. (FINAL LIST normalized). Protein expression of all proteins normalized to the level of prelamin-A/C (found to be the most stable expressed protein without differences between groups in raw data), as described in the method section are displayed. Mean and SEM are shown for patients with diabetes (T2DM) and non-diabetics (non-DM), as well as for type 2 diabetic treated with (plus metformin) or without metformin (no metformin). Fold change, F-test and p-tests are shown for comparisons between the groups.

D. (CV data). Data concerning analytical precision. For each of the ten runs A-J the ratio of the two samples nDM-pool (label 113) and DM-pool (label 114) was calculated and shown in the table. The mean, SD and the CV% for the ten repeat measurements are calculated for each identified and quantitated protein. The average CV% for all proteins were 10.4 % and 86 proteins had CV%’s less than 10 %.
**Table 3S Histomorphometric results.**

Quantitative histomorphometric analysis of the mammary artery from 30 type 2 diabetic and 30 non-diabetic individuals.

<table>
<thead>
<tr>
<th></th>
<th>Non-DM</th>
<th>T2DM</th>
<th>T2DM +Met</th>
<th>T2DM -Met</th>
<th>P-value</th>
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<tr>
<td>Mean</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
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<td>Collagen area fraction intima (%)</td>
<td>34.2</td>
<td>2.79</td>
<td>44.0</td>
<td>2.62</td>
<td>0.013</td>
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<tr>
<td>Collagen area fraction media (%)</td>
<td>44.6</td>
<td>3.44</td>
<td>53.8</td>
<td>3.22</td>
<td>0.055</td>
</tr>
<tr>
<td>Collagen area fraction adventitia (%)</td>
<td>54.5</td>
<td>2.76</td>
<td>58.6</td>
<td>2.18</td>
<td>0.249</td>
</tr>
<tr>
<td>α-actin pos cells intima (n/µm²) *10⁻⁵</td>
<td>107</td>
<td>11.3</td>
<td>87.7</td>
<td>9.15</td>
<td>0.181</td>
</tr>
<tr>
<td>α-actin pos cells media (n/µm²) *10⁻⁵</td>
<td>215</td>
<td>15.5</td>
<td>188</td>
<td>20.4</td>
<td>0.287</td>
</tr>
<tr>
<td>α-actin pos cells adven. (n/µm²) *10⁻⁵</td>
<td>108</td>
<td>10</td>
<td>70.0</td>
<td>9.94</td>
<td>0.006</td>
</tr>
<tr>
<td>Elastin area fraction intima (%)</td>
<td>66.4</td>
<td>1.8</td>
<td>67.4</td>
<td>1.6</td>
<td>0.683</td>
</tr>
<tr>
<td>Elastin area fraction media (%)</td>
<td>29</td>
<td>3.2</td>
<td>22.6</td>
<td>2.6</td>
<td>0.127</td>
</tr>
<tr>
<td>Elastin area fraction adventitia (%)</td>
<td>48.2</td>
<td>1.7</td>
<td>48.4</td>
<td>1.4</td>
<td>0.932</td>
</tr>
<tr>
<td>Macrophage infiltration (n/µm²)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.269</td>
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</table>
Supplemental Figure SF1. All histological images for histomorphometry.

Images are attached as an extra pdf-file containing all images: CD 68 staining, Weigert, Masson and Smooth muscle alpha-actin staining for all involved patients (type 2 diabetes: D1-D30 and non-D: C1-C30). Labeling of samples (T2DM: D1-D30, and non-DM: C1-C30) is the same as for the proteome labelling. Weigert, Mason and Smooth Muscle Staining contains the demarcation lines (made by an investigator unaware of disease and medication status), demarking the different areas.
CD 68 staining
Weigert staining
Masson staining
Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
Weigert staining
Masson staining
Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining
Weigert staining
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Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining
Weigert staining
Masson staining
Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

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Smooth muscle α-actin staining
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Smooth muscle α-actin staining
Weigert staining

Masson staining

Smooth muscle α-actin staining
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Masson staining
Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
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Smooth muscle α-actin staining
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Weigert staining
Masson staining
Smooth muscle α-actin staining
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Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining

Weigert staining

Masson staining

Smooth muscle α-actin staining
CD 68 staining
Weigert staining
Masson staining
Smooth muscle α-actin staining