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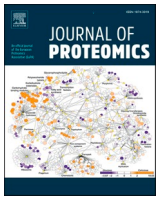

A targeted multiplex mass spectrometry method for quantitation of abundant matrix and cellular proteins in formalin-fixed paraffin embedded arterial tissue

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ABSTRACT

Assessment of proteins in formalin-fixed paraffin-embedded (FFPE) tissue traditionally hinges on immunohistochemistry and immunoblotting. These methods are far from optimal for quantitative studies and not suitable for large-scale testing of multiple protein panels. In this study, we developed and optimised a novel targeted isotope dilution mass spectrometry (MS)-based method for FFPE samples, designed to quantitate 17 matrix and cytosolic proteins abundantly present in arterial tissue. Our new method was developed on FFPE human tissue samples of the internal thoracic artery obtained from coronary artery bypass graft (CABG) operations. The workflow has a limit of 60 samples per day. Assay precision improved by normalisation to both beta-actin and smooth muscle actin with inter-assay coefficients of variation (CV) ranging from 5.3% to 31.9%. To demonstrate clinical utility of the assay we analysed 40 FFPE artery specimens from two groups of patients with or without type 2 diabetes. We observed increased levels of collagen type IV α1 and α2 in patients with diabetes. The assay is scalable for larger cohorts and advantageous for pathophysiological studies in diabetes and the method is easily convertible to analysis of other proteins in FFPE artery samples.

Significance: This article presents a novel robust and precise targeted mass spectrometry assay for relative quantitation of a panel of abundant matrix and cellular arterial proteins in archived formalin-fixed paraffin-embedded arterial samples. We demonstrate its utility in pathophysiological studies of cardiovascular disease in diabetes.

1. Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue is the preferred long-term storage method for tissue samples due to its ability to preserve intact tissue structures and proteins. Moreover, archiving tissue samples in FFPE blocks provide easy handling at room temperature and accessibility for further preparation of tissue specimens for downstream analyses [1,2]. FFPE tissue is mostly used for conventional histology and immunohistochemistry (IHC) including detection and quantitation of proteins and nucleic acids (RNA and DNA) [3–9]. While FFPE tissue is widely used in routine pathology it also plays an important role as a source of bio-specimen in clinical studies [10–14]. The use of such stored tissue combined with clinical information is a valuable resource for both retrospective and prospective human studies [15].

Traditional methods for protein analysis in FFPE tissue include IHC and Western blotting [11]. While the latter methods require protein solubilisation and size-separation by gel electrophoresis prior to immune detection, the former is based on direct recognition of binding epitopes on the analyte by IHC antibody reagents. Thus, due to low solubility of matrix proteins, detection and quantitation of these proteins are often carried out using IHC [16,17]. Unfortunately, IHC does not support higher level of multiplexing and quantitation is at best only semi-quantitative under optimal conditions [18]. Moreover, in large-scale testing of biobanks in retrospective studies and even with multi-

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parameter fluorescence detection, multiplex capabilities of IHC are limited [19].

A growing number of studies have demonstrated the feasibility of multiple reaction monitoring-mass spectrometry (MRM-MS) for targeted quantitative proteomic analyses of various FFPE-based tissue specimens [20–22]. Despite the fact that protein localisation in the microenvironment is lost using this method, MRM-MS has many advantages when quantitative analyses is needed, as it enables multiplexing and incorporates internal isotopic standards, ensuring minimal influence from extraction variability. Moreover, it is precise, specific, and standardisable and can even be harmonised across laboratories [23–25].

While optimisation and comparisons of FFPE MRM-MS protocols have focused on soluble proteins from various tissues and plasma [26,27], no targeted methods have yet been described for extracellular matrix proteins in connective tissue. Such a tool will be of great value for example in pathophysiological studies of the arterial protein composition in atherosclerosis and aneurysms, as well as in hypertension, arterial stiffness and diabetic macroangiopathy [28]. To this end, we have therefore developed and validated a robust and precise 15-plex MRM-MS assay for relative quantitation of mostly basement membrane and cytoskeletal proteins in FFPE artery samples.

2. Materials and methods

2.1. Arterial tissue samples

Human arterial tissue samples were obtained from patients undergoing coronary artery by-pass surgery at the Department of Vascular, Cardiac and Thoracic Surgery at Odense University Hospital (Odense, Denmark) from 2008 to 2018. As reported in previous studies, the samples are excess parts of the graft artery from the surgery (non-atherosclerotic internal thoracic artery) [29]. When performing the surgery, a small part of the artery is in excess (approx. 5 mm long) and collected and transferred to a formalin suspension (4% buffered paraformaldehyde) [30]. After fixation for 24–48 h at room temperature, the samples are embedded in paraffin for long-term preservation. The embedded samples are stored at room temperature as part of the Odense Artery Biobank (Odense, Denmark).

The MRM-MS protein assay was tested on 20 patients with type 2 diabetes mellitus (T2DM) and 20 patients without T2DM. The patients were predominantly male (17 males in diabetic group and 19 males in non-diabetic group) with a mean age of 70.5 years (67.5–73.5) for diabetic individuals and 66.8 years (63.2–70.4) for non-diabetic individuals.

2.2. Immunohistochemistry

Arterial FFPE samples were cut in 4 μm sections and stained by immunohistochemistry with antibodies against collagen type IV, alpha-1 (Wieslab AB MAB1), alpha-smooth muscle actin (Nordic Biosite Aps, #BSH-7459-1, clone BS66), h-caldesmon (DAKO #M3557, clone h-CD), desmin (Ventana Medical Systems #760–2513, clone DE-R-11) and myosin-11 (Ventana Medical Systems #760–760-2704) with a previously published protocol [29] using the UltraView Universal Horse

Table 1
Proteins and peptides included in the MRM-MS assay.

Protein	Protein type	Peptide[a, b]	Precursor ion *m/z* (+2)	Fragment ions m/z (+1)
Actin, aortic smooth muscle	Cellular (cytoskeletal)	VAPEEHPTLLTEAPLNPK	978.525	y5, y6, y12
		GYSFVTTAER	565.777	y5, y6, y8
Actin, cytoplasmic 1	Cellular (cytoskeletal)	VAPEEHPVLLTEAPLNPK	977.536	y3, y5, y12
		GYSFTTTAER	566.767	y5, y6, y8
Alpha-actinin 4	Cellular (cytoskeletal)	LASDLLEWIR	608.340	y3, y5, y8
		TINEVENQILTR	715.386	y3, y7, y8
Beta-galactosidase[c]	E.coli protein, trypsin-digestion control	LWSAEIPNLYR	681.364	y5, y6, y9
		VNWLGLGPQENYPDR	884.438	y3, y9, y11
Biglycan	Extracellular matrix	VPSGLPDLK	463.271	y4, y6, y7
Caldesmon	Cellular (actin-binding)	LEQYTSAIEGTK	670.341	y7, y8, y9
		INEWLTK	452.250	y3, y4, y5
Collagen IV, alpha-1 chain	Extracellular matrix	GFP[*OH*]GTSLP[OH]GPSGR	632.320	y6, y8, y11
Collagen IV, alpha-2 chain	Extracellular matrix	IAVQPGTVGPQGR	640.359	y5, y6, y9
		GGVSAVPGFR	473,759	y4, y5, y7
Desmin	Cellular (intermediate filament)	VELQELNDR	558.288	y5, y6, y7
		VAELYEEELR	625.817	y5, y6, y7
Fibulin 5	Extracellular matrix	CEEPYLR	483.721	y3, y4, y5
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Cellular (glycolysis enzyme)	GALQNIIPASTGAAK	706.399	y8, y9, y11
		LISWYDNEFGYSNR	882.405	y3, y5, y6
Laminin, gamma 1	Extracellular matrix	SYYYAISDFAVGGR	784.873	y8, y9, y10
		LIEIASR	401.245	y4, y5
Myosin 9	Cellular (motor protein)	VVFQEFR	462,751	y3, y4, y5
		YEILTPNSIPK	637.853	y6, y7, y8
Myosin 11	Cellular (motor protein)	LVWVPSEK	479.274	y4, y6, y7
		DTSITGELEK	546.775	y5, y6, y8
Perlecan	Extracellular matrix	VDSYGGSLR	477.238	y5, y6, y7
		SPAYTLVWTR	597.319	y4, y5, y6
		LEGDTLIIPR	563.827	y4, y6, y8
Tubulin, alpha-1a	Cellular (microtubular)	DVNAAIATIK	508.293	y5, y6, y8
		EIIDLVLDR	543.314	y3, y4, y6
		AVFVDLEPTVIDEVR	851.457	y8, y9, y11
Vimentin	Cellular (intermediate filament)	FADLSEAANR	547.267	y5, y6, y8
		EYQDLLNVK	561.295	y3, y4, y6
Vinculin	Cellular (cytoskeletal)	DYLIDGSR	469.733	y4, y5, y6
		STVEGIQASVK	559.806	y7, y8, y9
		ELTPQVVSAAR	585.827	y5, y6, y8

[a] All peptides were analysed along with a matching heavy $^{13}C/^{15}N$- lysine (+8 Da) or arginine (+10 Da) stable isotope labelled synthetic peptide standard.
[b] Peptides containing cysteine carry the fixed modification carboxyamidomethyl (57.02 Da) due to iodoacetamide alkylation.
[c] Beta-galactosidase was used as a spike-in control for monitoring sample recovery and digestion efficacy.

Radish Peroxidase (HRP) Detection Kit from Ventana Medical Systems. The protocol for all antibodies was optimised in relation to antibody dilution, pretreatment of tissue etc. using a multiblock including >15 different tissues.

2.3. Protein and peptide selection

For our multiplex assay, 17 human proteins were selected. We focused on basement membrane proteins i.e. collagen type IV as well as other extra cellular matrix and structural cellular proteins abundantly present in arterial tissue (Table 1). For each protein, one, two or three proteotypic peptides were identified as stable analytes after in silico digestion and iterative analysis of arterial extracts. For each peptide, sequence uniqueness was verified using BLASTP [30]. Due to heavy proline hydroxylation of the type IV collagen α1 chain, we included a stable modified dual hydroxyproline-containing peptide for quantitative analysis (Table 1). Furthermore, we used *E. coli* beta-galactosidase (LacZ) as internal sample digestion control.

2.4. Deparaffinisation of FFPE samples

For analysis, five slices of tissue (each 10 μm thick) were cut using a Microtome and placed into a 1.5 ml tube (Eppendorf, Hamburg, Germany) totaling 50 μm of tissue in one sample. The tubes were then stored at −80 °C for optimal preservation of proteins.

Samples were treated with 320 μl of deparaffinisation solution (Qiagen Deparaffinisation Solution, Qiagen, Hilden, Germany) and heated for 10 min at 56 °C while shaking at 1200 rounds per minute (rpm) as indicated by the manufacturer. Stepwise handling of samples is illustrated in graphical abstract. Finally, the sample was centrifuged at 17000 ×*g* for 5 min. After deparaffinisation and careful removal of excess solution, the tissue was washed once with 1 ml of demineralised water while heating for 10 min at 56 °C and shaking at 1200 rpm. Before removing the supernatant, the sample was centrifuged for 10 min at 17000 ×*g*.

2.5. Protein extraction of FFPE samples

The deparaffinised tissue was solubilised with 50 μl extraction buffer (20 mM Tris pH 8.8, 50 mM DTT, 1% octyl-β-D-glycoside, 5% sodium deoxycholate [DOC]) including 2 μg LacZ digestion control (Sigma-Aldrich). Extracted proteins were subsequently transferred to 0.2 ml PCR tubes and denatured for 60 min in a thermocycler (20 min at 99 °C and 40 min at 80 °C).

2.6. Protein precipitation

Proteins were precipitated using 150 μl 25% trichloracetic acid (TCA) and samples were subsequently centrifuged for 20 min at 4 °C, 20817 ×g. Pellets of protein, tissue, and DOC were dissolved/washed in 200 μl acetone by incubation and shaking at 37 °C, 1200 rpm for 10 min. After centrifugation for 10 min, pellets were washed once more with acetone and left to air dry for 15 min.

2.7. Protein digestion

Protein/tissue pellets were resuspended and denatured in 5 μl urea (8 M) for 10 min at room temperature. Samples were reduced by addition of 35 μl DTT (10 mM DTT in 50 mM ammonium bicarbonate) and incubated for 20 min at room temperature. Next, samples were alkylated by addition of 5 μl iodacetamide (0.2 M) following incubation protected against light for 20 min at room temperature. Finally, proteins were digested using trypsin 0.2 μg (Sequencing Grade Modified Trypsin, Promega) for 18 h at 37 °C while shaking at 400 rpm. After incubation, samples were acidified in formic acid (1% final concentration), centrifuged 5 min at 17000 ×*g*, transferred to new 0.5 ml tubes and stored frozen at −20 °C prior to MRM-MS analysis.

2.8. Synthetic internal standard peptides

A balanced mix of synthetic internal standard (SIS) peptides were added to each sample, aiming at a ratio of endogenous peptide to SIS peptide of 1:1 and ranging between 0.01 and 3.6 pmol per sample. Thus, the amount of SIS peptide added correlates to the amount of endogenous peptide present in the sample. For the present multiplex assay, a pool of 34 SIS peptides in 0.1% FA, 2% acetonitrile (Table 1) (JPT Peptide Technologies GmbH) was mixed and stored frozen at −80 °C before use.

2.9. Liquid chromatography coupled with multiple-reaction-monitoring mass spectrometry

Peptide samples (5 μl sample, 5 μl SIS peptide pool and 10 μl formic acid 0.1%), were extracted using Evotip disposable trap columns (Evosep Biosystems) according to the manufacturer's instructions [31] and analysed using an integrated microflow Evosep One HPLC/TSQ Altis triple quadrupole mass spectrometry system equipped with an EASY-Spray ion source (Evosep Biosystems and Thermo Fisher Scientific). The Evosep One was mounted with an 8 cm analytical column and samples were analysed in scheduled MRM mode using a 60 samples per day LC method. The analytical separation runs for 21 min on a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), according to the preset Evosep program. The spray voltage was static (1800 V) and resolution was set to 0.7 Da full width at half maximum for Q1 and Q3 mass filters. Collision gas pressure was 1 mTorr. The final method had a cycle time of 1.5 min for 214 transitions (endogenous + SIS peptides, Table 1).

2.10. Data analysis

All MS raw files were processed in Pinpoint 1.4 (Thermo Fisher Scientific) and exported as ratio data (peak area ratio of endogenous to SIS peptide, light to heavy, L/H). Further normalisation and statistical analysis was performed using Microsoft Excel 2016 (Microsoft Corporation) and Stata 16 (StataCorp LLC). Mann Whitney *U* test was used for non-parametric statistics and Pearson correlation analysis for linear association between protein variables. For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

To explore the possibility of establishing a multiplex protein assay for monitoring pathophysiological alterations in extracellular matrix filled FFPE-artery specimens, we initially compiled a list of basement membrane and cytoskeleton proteins and derived tryptic peptides for unscheduled MRM analyses. For collagen type IV, the high content of proline hydroxylation required further optimisation by identification of specific tryptic peptides and their hydroxylation levels. For the alpha-1 and alpha-2 chains we identified the partially hydroxylated peptide R/GFP[*OH*]GTSLP[OH]GPSGR and the non-hydroxylated peptides R/IAVQPGTVGPQGR and R/GGVSAVPGFR, respectively, as surrogate markers of collagen type IV (Table 1). We validated all targeted peptides by stable-isotope-dilution of co-eluting heavy peptides standards (Table 1 and Supplementary Fig. S1). Next, we optimised and evaluated all steps in the final protocol (Fig. 1A) for optimal protein extraction efficacy and reproducibility. By initial comparison of multiple detergents for protein extraction from FFPE-arteries, we determined that the combination of the two detergents octyl-β-D-glycoside and sodium deoxycholate provided consistent and high peptide signals (total ion current) in the MRM assay. In this setting, sonication steps did not improve the extraction efficiency. Finally, we established LC-MS procedures for the included peptides such that FFPE-artery samples could be analysed in scheduled MRM mode with a 60 samples/day analysis

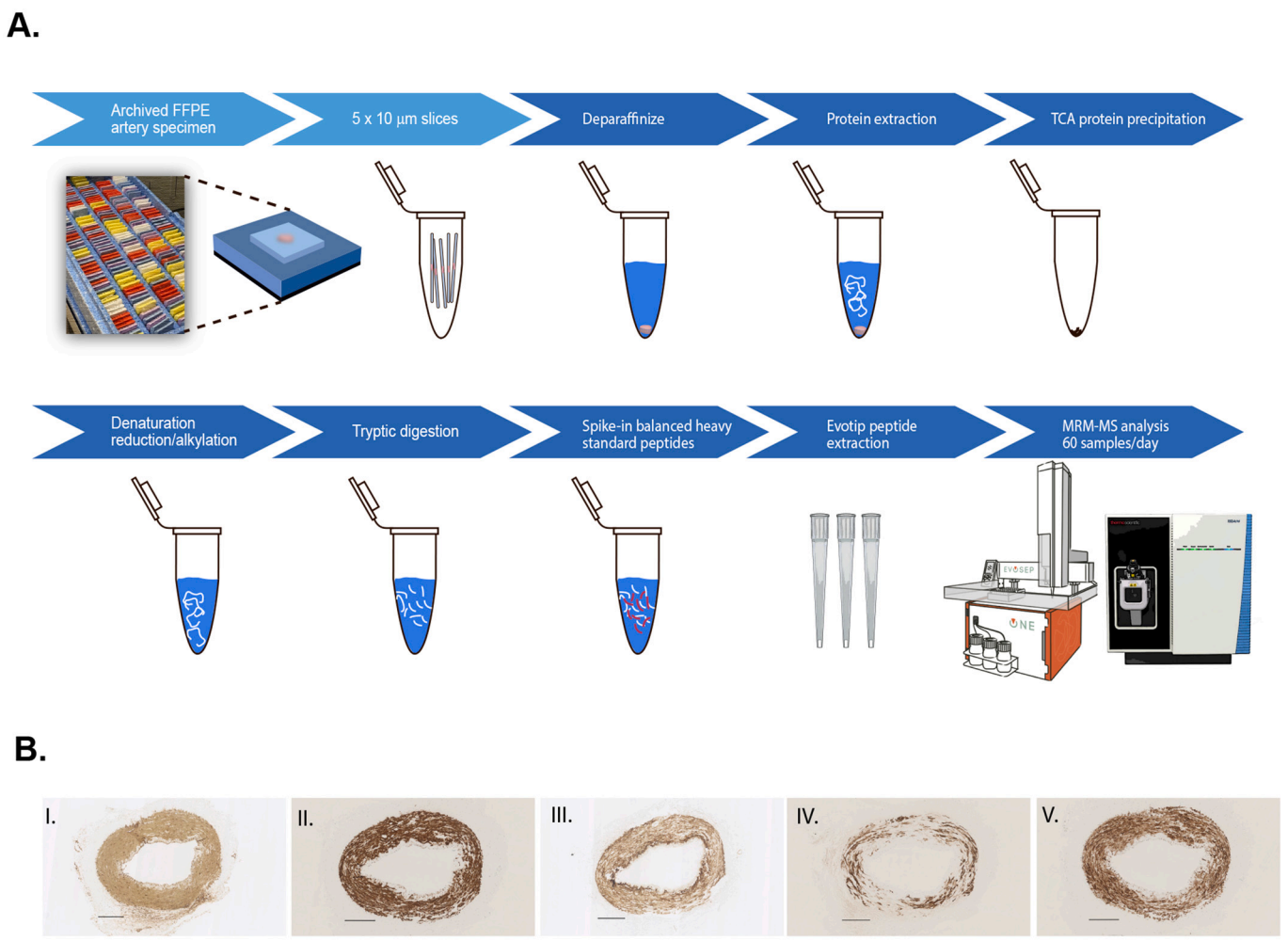

Fig. 1. Flow diagram of the biochemical quantification method and structure of internal thoracic artery specimens. A. Flow diagram of artery processing, protein extraction/digestion and MRM-MS analysis. B. Immunohistochemically visualization of the internal thoracic artery stained with antibodies against five of the proteins analysed in the MRM assay, i.e. alpha-smooth muscle actin, caldesmon, collagen type IV-alpha 1, desmin and myosin-11. Scalebars indicate 250 µm.

cycle. The final optimised protocol for the FFPE MRM-MS workflow is shown in graphical abstract.

For quantitation of all targeted peptides, we established linearity of measurements by performing dilution series of stable isotope standard pools spiked into FFPE-artery peptide preparations (Supplementary Fig. 1). All measured assay peptides (light to heavy ratios, L/H) were within the linear range of each serial dilution.

The final assay consists of 34 tryptic peptides representing 17 proteins (basement membrane proteins, other matrix components and cellular proteins including cytoplasmic 1 (beta) actin and aortic smooth muscle actin for normalisation). In addition, we used LacZ as an internal spike-in control protein to monitor variation in protein recovery and digestion efficiency between samples. If LacZ levels indicated <50% digestion efficiency, the sample in question was discarded and replaced with a new tissue sample from the same patient.

After optimisation, the FFPE-artery samples could be analysed in scheduled MRM mode on an Evosep One-TSQ Altis LC-MS system using a 21-min preformed gradient (60 samples/day analysis cycle).

The precision profile of the assay was determined by analysis of triplicate sample preparations of FFPE-artery slices from three patients, i.e. three tubes with slices from the same artery-containing paraffin block were prepared from each of three patients, followed by extractions and LC-MS analysis on three different days. The intra- and inter-assay (between days) CVs in the crude MRM dataset were 16.4–30.7% and 21.9–41.8%, respectively (Table 2), while normalised MRM data improved CVs (3.4–21.7% and 6.6–31.9%), respectively (Table 2).

A panel of MRM-measured proteins were visualized by immunohistochemistry showing the structure of the arteries (Fig. 1B). The internal thoracic artery is a typical muscular artery with a thin tunica intima consisting of endothelial cells and a thicker matrix-filled tunica media where most of the proteins are situated either in smooth muscle cells (aortic smooth muscle actin, desmin, caldesmon, myosin-11) or in the surrounding matrix (collagen type IV-alpha 1). Beneath these layers are the tunica adventitia, which only contain small amounts of the displayed proteins.

Univariate correlation analysis showed that the measured amounts of collagen type IV, alpha-1 chain correlated very well to both the alpha-2 chain of collagen type IV and laminin, but not to GAPDH (Fig. 2).

To test the MRM assay in a clinical context, we compared 20 FFPE samples from diabetes patients with 20 non-diabetic controls. We found a significant increase in collagen type IV in patients with type 2 diabetes (median normalised area ratio for α1: 1.17 vs. 0.88, $p = 0.0004$, for α2: 1.13 vs. 0.89, $p = 0.034$) (Fig. 3). Furthermore, actin (cytoplasmic 1) was significantly increased in the diabetic group (median normalised area ratio 4.33 vs. 4.02, $p = 0.04$) as well as perlecan (median normalised area ratio 1.41 vs. 1.00, $p = 0.04$). We also saw a high level of correlation between collagen type IV, α1 and α2 subunits ($r = 0.973$, $p > 0.005$, non-normalised data). None of the other assay proteins differed significantly between the groups (Supplementary Table 1). For two proteins (tubulin alpha 1 and myosin 9) two outliers (area ratio > 7)

Table 2
Inter-assay and intra-assay imprecision for all measured proteins depending on normalisation status.

	Non-normalised		Normalised to actin (cytoplasmic 1)		Normalised to actin (aortic smooth muscle)	
	Inter-assay CV%	Intra-assay CV%	Inter-assay CV%	Intra-assay CV%	Inter-assay CV%	Intra-assay CV%
Actin, aortic smooth muscle	22.2	20.5	5.3	3.1	NR	NR
Actin, cytoplasmic 1	22.3	19.7	NR	NR	6.6	3.4
Alpha-actinin 4	41.8	30.7	31.2	17.3	31.9	18.2
Beta-galactosidase[a]	21.9	16.4	26.5	21.7	25.4	21.7
Biglycan	35.8	26.9	23.7	11.6	24.5	11.5
Caldesmon	25.5	23.0	11.8	10.4	11.9	9.2
Collagen IV, alpha-1 chain	26.6	25.0	14.8	14.0	14.7	12.6
Collagen IV, alpha-2 chain	25.8	23.2	13.2	12.7	13.5	11.5
Desmin	38.3	25.8	27.8	10.0	28.4	8.9
Fibulin-5	38.0	22.5	28.9	12.6	29.8	11.1
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	29.7	25.5	12.4	10.3	19.4	13.1
Laminin, gamma 1	24.1	23.0	13.3	11.6	13.0	9.8
Myosin 9	27.2	24.9	15.4	12.8	18.4	15.2
Myosin 11	27.5	26.3	14.0	14.1	13.5	12.5
Perlecan	25.9	25.6	14.4	13.2	13.5	10.9
Tubulin, alpha-1a	35.2	29.4	19.4	15.0	25.5	17.8
Vimentin	27.4	22.9	11.7	7.4	12.3	5.8
Vinculin	27.7	24.0	14.7	11.0	15.1	8.7

CV%: coefficient of variation. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. NR = not relevant.
[a] Beta-galactosidase was used as a spike-in control for monitoring sample recovery and digestion efficacy.

were removed. This did not alter significance levels.

4. Discussion

Human arterial tissue is traditionally analysed using imaging or semi-quantitative techniques such as immunohistochemical staining or western blotting. These techniques lack multiplexing capability as well as quantitative sensitivity and specificity. The structural properties of arterial tissues (i.e. high content of smooth muscle cells and extracellular matrix) and low protein solubility make traditional techniques challenging to use in large scale analyses.

Here we present a novel optimised protocol for MRM-MS analysis of artery proteins from FFPE tissue. To the best of our knowledge, this is the first MS-based method described for targeted multiplex quantitation of proteins in archived FFPE artery tissue. Our development of this technique has led to higher precision in quantitation of arterial tissue proteins coupled with easier preparation for high throughput analysis. We have shown that this method can be applied in a clinical setting to detect pathophysiological differences in patients, i.e. higher levels of collagen IV in patients with diabetes (Fig. 3).

During the method development stage, we performed optimisation of a number of steps. First, tissue deparaffinisation had to be effective in order to eliminate its interference and incompatibility with downstream reagents used. Traditional deparaffinising agents such as xylene are environmentally hazardous and difficult to handle. We applied a commercial xylene-free deparaffinising agent with satisfactory results. Second, we evaluated a number of protein extraction buffers for efficiency and compatibility with MRM-MS analysis. To this end, we identified a combination of the two detergents octyl-β-D-glycoside and sodium deoxycholate as optimal for artery protein solubilisation and extraction. This, in combination with extended heating and subsequent protein precipitation, provided an optimal preparation prior to standard proteomics sample handling (i.e. protein denaturation/reduction/alkylation and tryptic proteolysis). Third, variations due to heterogeneity of the tissue samples, variability in protein extraction and resulting unequal total protein in the samples was overcome using internal normalisation to actin. Prompted by the high assay variation presumably due to comparison of different slices from the same patient and possibly due to variation in protein extraction between samples, we investigated the use of actin normalisation. Actins are often used as internal controls because of high abundancy and low variations in many disease situations. Applying both actin (cytoplasmic 1) and aortic smooth muscle actin as internal controls strongly reduced the variation, as both intra- and inter-assay CV's improved significantly in normalised MRM data. Moreover, we included a spike-in protein to monitor protein recovery and digestion efficiency across samples, an important quality control when FFPE material is analysed. Overall, our FFPE artery MRM-MS method is fit for purpose for analysis of FFPE tissue samples. While the initial step of deparaffinisation currently requires sample handling in tubes, the remaining protocol can be handled in tubes or 96-well plate format and is compatible with the 60 samples/day LC-MS method. Detection and quantitation of low-abundant proteins and level of precision is superior

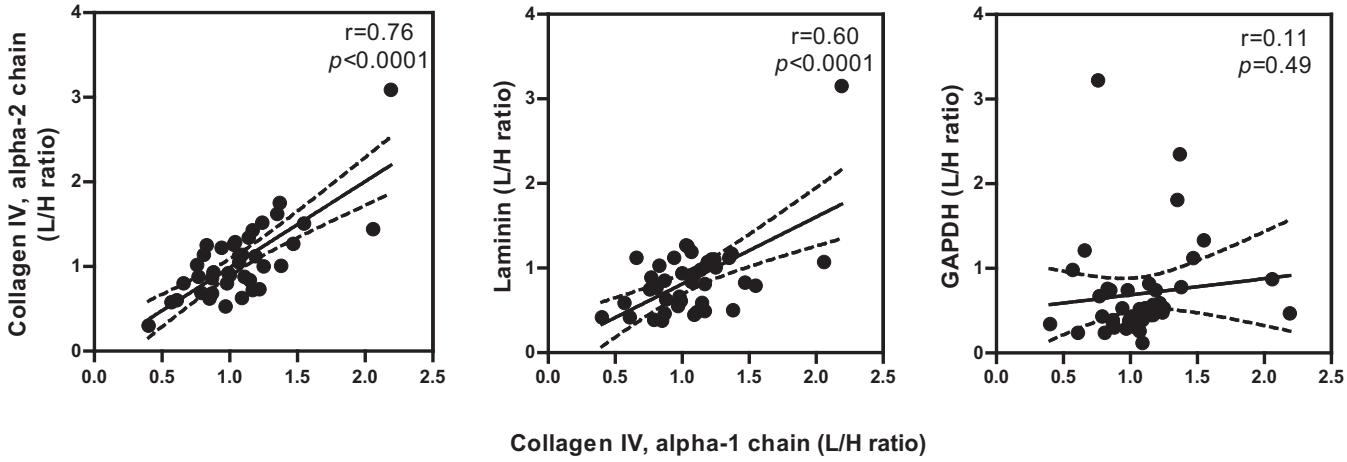

Fig. 2. Associations between collagen type IV, alpha-1 and alpha-2, laminin and GAPDH.
Correlations between the arterial content of collagen IV, alpha-1 chain and three other proteins measured in the MRM assay: collagen IV, alpha-2, laminin and GAPDH. Lines show regression with calculated Pearson r- and *p*-values, as indicated.

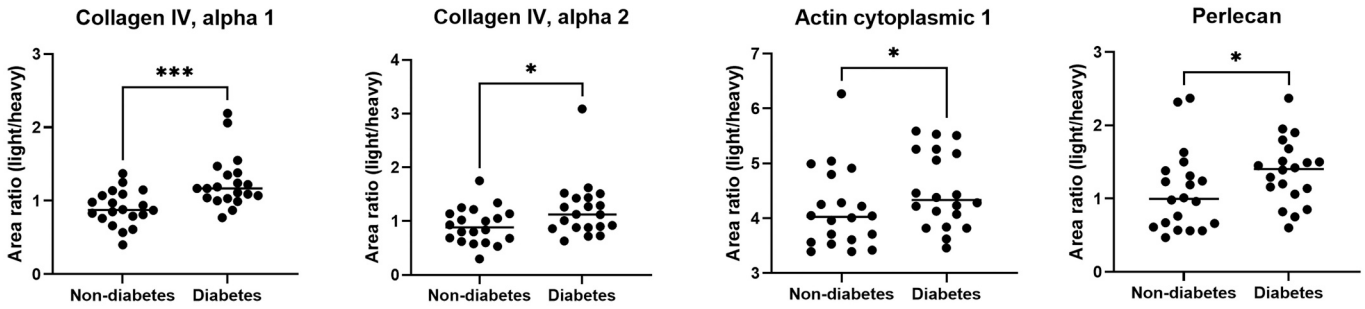

Fig. 3. Protein levels in patients with and without type 2 diabetes. Light/heavy (L/H) area ratio from 20 patients with type 2 diabetes and 20 non-diabetics, as indicated. Data was normalised to actin, aortic smooth muscle. Horizontal lines indicate median values and minor horizontal short lines indicate 95% confidence interval. * indicates $p < 0.05$ (Mann Whitney, diabetes versus non-diabetes group). *** indicates $p < 0.000$ (Mann Whitney, diabetes versus non-diabetes group).

to traditional methods.

The main purpose of our study was to develop a multiplex protein assay for quantification of especially extracellular matrix proteins in a matrix filled sample, in this case arteries. Optimally, our matrix-focused method should be compared to other tests for this purpose. However, although immunohistochemical staining may provide information on the distribution of the proteins, any attempt to make quantitative measurements of either staining intensity or area are notoriously imprecise and difficult to interpret in relation to the actual protein concentrations in the tissue. Likewise, western blotting of matrix molecules are problematic to compare our MRM-based results to, since it demands extractions, which may cause degradation, making antibody-detection unreliable. Nevertheless, we believe that our inter-protein-correlations underline the reliability of our findings since the results makes biological sense: the two types of collagen type IV, i.e. alpha-1 and alpha-2 are at the RNA level produced at equal amounts from two genes with a common promotor [32]. This fits with our finding of a very high correlation between the amounts of the proteins at the tissue level. Moreover, laminin is present in basement membranes, as is collagen type IV, compatible with their high correlations, whereas GAPDH is found in all cells, including cells in various amounts of tunica adventita present in the samples, and it is therefore not surprising that there is no correlation between the concentrations of this proteins and collagen type IV, which is only present around smooth muscle cells in the tunica media. Taken together, these observations strengthen the credibility of results obtained by the method presented.

Application of the method to FFPE artery samples demonstrated increased levels of type IV collagen in patients with type 2 diabetes compared to non-diabetic controls (Fig. 3). This result was in line with previous untargeted proteome analysis by Preil and coworkers [29] and in another way highlight the usefulness of the method to find even smaller differences in challenging tissue samples.

5. Conclusions

We have developed a novel and precise MRM-MS assay for multiplex relative quantitation of abundant matrix and cellular proteins in human arterial FFPE samples. We provide an optimised protocol for sample preparation and handling of FFPE tissue coupled to MRM-MS analysis. Finally, we have demonstrated clinical application of the assay showing diabetes associated differences in collagen type IV in patient samples.

6. Future aspects

Based on the need for investigations of tissue proteins in the future, we foresee that our results, which demonstrate precision, robustness and large capacity, point towards the use of MRM-MS for tissue studies in the future. The MRM-MS method can, with some moderations, be applied on different types of tissue and include various proteins, making it versatile and applicable in multiple research settings.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104775.

Ethics approval and consent to participate

This study is part of the Odense Artery Biobank, approved by the Regional Committee on Health Research Ethics for Southern Denmark (case number S-20140202). All participants have received oral and written information of the study. All participants have signed written consent forms. Participants can withdraw their consent at any given time. This study was performed in accordance with the Declaration of Helsinki.

Author contributions

AFT performed tissue analysis, data processing and was responsible for the main writing process. LPR included patients and assisted in revision of the paper. LMR performed study design, data analysis as well as review of the paper. MO performed method development and provided technical support in analysis of data and statistical analysis. All authors read and approved the final paper.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

The datasets generated and analysed in this study are available in the Supplementary Table 1.

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