ORIGINAL ARTICLE

Raman spectroscopy reveals collagen and phospholipids as major components of hyalinosis in the arteriolosclerotic ulcer of Martorell

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Abstract

Background Arteriolosclerotic ulcers of Martorell are histologically characterized by hyaline arteriolosclerosis resulting in concentric occlusion of the arteriolar lumina. Although several authors have previously reported on hyaline changes in hypertensive arteriolopathies, so far, little information is available on the molecular composition of hyaline wall depositions.

Objectives This study aimed at the molecular characterization of hyaline arteriolar deposits in patients with hypertensive arteriolopathy using confocal Raman spectroscopy.

Methods Samples of patients diagnosed with arteriolosclerotic ulcers of Martorell were analysed using confocal Raman spectroscopy. The findings were correlated with histological analyses. Skin samples from healthy, non-hypertensive patients served as controls.

Results Confocal Raman spectroscopy analysis revealed that subendothelial hyaline deposits in arteriolosclerotic ulcers are mainly composed of collagen and phospholipids, in particular phosphatidylcholine. The presence of collagen within hyaline deposits was confirmed by Masson’s Trichrome and Picrosirius Red staining. Additionally, the presence of collagen could also be shown for hypertensive nephrosclerosis. Actin was markedly decreased in hyalinized compared to control vessels, corresponding to the loss of smooth muscle cells in the process of hyalinization. This was confirmed by immunofluorescence staining for α-smooth muscle actin and desmin.

Conclusion The present findings suggest that arteriolar hyaline deposits in hypertensive arteriolopathy are mainly composed of collagen and phospholipids, in particular phosphatidylcholine. Together with the concurrent absence of actin, these findings suggest that potentially critical disease mechanisms involve pressure-induced vascular smooth muscle cell apoptosis with subsequent deposition of collagen.

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Conflict of interest

Authors declare that they have no conflict of interest to declare.

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Introduction

Arteriolosclerotic ulcers of Martorell (ASUM) are characterized by disproportional pain, a predilection site on the dorsolateral aspect of the lower leg, and are associated with long-standing arterial diastolic hypertension.1–3 Epidemiological data are scarce; however, these ulcers are estimated to account for up to 15% of leg ulcers in specialized wound centres, even if their overall share in all types of leg ulcers is estimated to be much lower.4,5 Currently, gold standard treatment comprises antihypertensive therapy and necrosectomy followed by split-thickness skin grafting.5,6 The presence of hyaline arteriolosclerosis with or without media calcinosis in arterioles of the lower dermis and

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subcutis has been described as its histological hallmark.7–11 This microvascular pathology involves hardening and loss of elasticity of the arteriolar wall and is commonly associated with chronic hypertension and/or diabetes mellitus.12,13 Histologically, it is characterized by ‘hyalinosis’ in the media of arteriolar vessels that can be described as a subendothelial accumulation of a large amount of amorphous, cosinophilic, glassy material.14 Due to progressive thickening of the arteriolar vessel wall and subsequent luminal narrowing, a significant increase in the minimum vascular resistance and reduced capillary perfusion pressure can be observed.5 If the decrease in luminal diameter leads to luminal stenosis, local ischaemic injury consequently develops, which can manifest in ischaemic skin infarction, such as in ASUM.11 Hyalinosis is often accompanied by a loss of smooth muscle cells, whereas the endothelial lining mostly remains intact.15 Additionally, histological analysis often shows periarteriolar inflammatory changes mainly characterized by perivascular neutrophil infiltrates and fibrin deposits.5,9,16 Besides arteriosclerotic ulcers,5,17–19 hyaline arteriolosclerosis can also be observed in hypertensive nephrosclerosis resulting in a decline in renal function.14,20 Renal insufficiency secondary to essential hypertension is the second most common cause of end-stage renal disease after diabetes mellitus, and its prevalence is increasing worldwide.20,21 Hypertensive kidney disease is histologically characterized by nephroangiosclerosis, implicating intimal hyperplasia, arteriolar hyalinosis and smooth muscle cell hypertrophy in medium- and small-sized vessels that entail glomerular damage.20,22 Vascular hyalinosis has been shown to be negatively correlated with eGFR levels and renal function outcomes and is a potential surrogate marker for interstitial hypoxia.23,24 Additionally, hyaline arteriolosclerosis can also be found in diabetic nephropathy or renal transplant recipients receiving calcineurin inhibitors.20,25–27 It has also been described in diabetic retinopathy, placental blood vessels of hypertensive women and subcortical arteriosclerotic encephalopathy.17–19

Although several authors have previously reported on hyaline changes in hypertensive arteriopathies,9,20,28 so far, only scarce information is available regarding the actual molecular composition of the hyaline wall depositional. However, this information is of major importance with regard to the development of potential therapeutic modulations of these vascular changes. Therefore, this study aimed at the molecular characterization of arteriolar hyaline deposits of cutaneous dermo-hypodermal arteriolopathy using confocal Raman spectroscopy, which has recently received increasing attention in the biomedical field, as it offers the ability to characterize the molecular composition of biological tissues at the micro- and nano-levels.29,30

**Materials and methods**

The study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki31 and in accordance with Austrian law. Study protocols and patient enrolment were formally approved by the local ethics committee of the Medical University of Vienna (No. 1171/2017).

Samples of patients diagnosed with ASUM and hypertensive nephropathy at the University Hospital of Vienna, Austria, were included in the study. In addition, healthy skin samples were used as controls.

**Confocal Raman microscopy**

Raman measurements of arteriolar vessels with and without hyalinosis found in skin biopsies taken from ASUM and arteriolar vessels from non-hypertensive controls were performed using a confocal Raman microscope (Alpha300RA, WITec GmbH, Ulm, Germany; n = 3 arterioles). The excitation light source for all measurements was a linearly polarized (0°) coherent compass sapphire VIS laser (λex = 532 nm, laser power = 44 mW; WITec GmbH) focussed through a 100× oil immersion objective (numerical aperture = 1.4, coverslip corrected 0.17 mm; Carl Zeiss, Jena, Germany) onto the sample. The backscattered Raman signal was directed through an optic multifibre (50 µm diameter) to a spectrometer (UHTS 300, WITec GmbH; 600 g/ mm grating) and finally to the CCD camera (Andor DU401 BV, Belfast, Northern Ireland). Tissue sections with 4 µm thickness were embedded in heavy water (D2O) and scanned with a lateral resolution of 0.3 µm by acquiring at every pixel one full wavenumber spectrum with an integration time of 0.133 s. The Control Four (WITec GmbH) acquisition software was used for Raman measurements.

**Data analysis**

Raman data analysis was performed using the WITec project FOURPlus 4.1 software (WITec GmbH, Ulm, Germany). Before the Raman images were generated, preprocessing was performed as follows: the scans were cropped, and a cosmic ray removal filter was applied. Non-negative matrix factorization (NMF) was carried out according to Prats-Mateu et al.32 with four to six endmembers. The endmember spectra reflecting the purest components with the strongest signal contributions were used in a basis analysis to fit the hyperspectral data set and visualize their distribution in the different histological sections. The extracted endmember and average spectra were baseline-corrected for better comparability. Additionally, a sum filter (band integration) was used to visualize the intensity of the Raman bands, which were assigned to specific molecular vibrations and components.

**Histochmical and immunofluorescence analysis**

Skin biopsy samples of ASUM, hypertensive nephropathy and healthy controls (n = 5 per group) were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections of 4 µm thickness were stained with H&E and Masson’s trichrome stain. Stained slides were scanned with Aperio ScanScope and analysed using Aperio ImageScope (Leica Biosystems, Wetzlar, Germany). Picrosirius red staining was performed to selectively
highlight collagen networks and analysed under polarized light using an AxiosImager (Carl Zeiss).33

Immunofluorescence staining was performed for the antigens alpha-smooth muscle actin (α-SMA, Cone: 1A4, Merck, Darmstadt, Germany) and desmin (Clone: D33, Dako, Glostrup, Denmark). For imaging analysis, AxiosImager and LSM 700 (Carl Zeiss GmbH, Oberkochen, Germany) were used.

Results
Staining and Raman measurements were performed on microsections of different representative arteriolar vessels from cutaneous biopsies of ASUM with and without histomorphological signs of hyalinosis (ASUM + hyalinosis and ASUM − hyalinosis) and arteriolar vessels from non-hypertensive controls (control). In Fig. 1a, the schematic shows the changes from a healthy arteriole to a hyalinized arteriole induced by shear stress. The deposition of hyaline material and smooth muscle cell apoptosis lead to a characteristic glassy appearance. This dense layer of subendothelial hyaline deposits appears in the H&E stained cross section as a pink homogenous area [Fig. 1b, (ASUM + hyalinosis)]. To reveal the micro-chemical differences between healthy and hyalinized arteriosclerotic arterioles, we performed correlative Raman measurements. In Fig. 1c, Raman images based on the CH stretch are displayed, showing all organic compounds at once. The hyalinized arteriole also shows this denser layer in the Raman image. However, the spectral signatures of the different layers have overlapping Raman bands; therefore, we used a multivariate unmixing algorithm to obtain the purest spectral signatures of the different components together with their distribution.

Distribution of three main components reveals ultrastructural differences
With the unmixing algorithm non-NMF, the most pure components were calculated from the control samples (Fig. 2a) and used in a second step to fit all hyperspectral data sets to compare the chemistry of the control and ASUM samples. The first spectrum (basis 1, blue) showed strong bands at 890, 1063 and 1133 cm⁻¹ (Fig. 2a), which were assigned to phospholipids according to previously reported spectra.34 The second spectrum (basis 2, red) was assigned to collagen as the most prominent bands at 851 and 937 cm⁻¹, 1256, 1456 and 1660 cm⁻¹ coincided with published collagen spectra.35–38 The third spectrum (basis 3, yellow) showed characteristic bands at 620, 640 and 1337 cm⁻¹, which were indicative of actin.39 The CH stretching vibrations from 2948 to 2982 cm⁻¹ were present in all three endmember spectra, as all three components had CH groups. The nearby broad band around 2490 cm⁻¹ was attributed to D₂O, as the samples were measured at D₂O (Fig. 2a).

The basis spectra of the three main components were used to fit all Raman hyperspectral data sets and visualize the distribution of phospholipids (blue), collagen (red) and actin (yellow, Fig. 2a). In the arteriolar vessels of the control samples, all

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**Figure 1** Correlative staining and Raman measurements of cross sections of representative arterioles. (a) Schematic representation of a healthy arteriole and an arteriosclerotic arteriole, with changes induced by continuously elevated shear stress, of the deep cutaneous plexus at the dermal/hypodermal junction. (b) Staining of cross sections, with haematoxylin and eosin (H&E), of arterioles of a non-hypertensive control (Control), a hyalinized arteriole (ASUM + Hyalinosis) and an arteriole without signs of hyalinosis (ASUM − Hyalinosis) from an ASUM sample. (c) Correlative Raman images based on the CH stretch (2800–3052 cm⁻¹) show all organic compounds. ASUM, Arteriolosclerotic ulcers of Martorell.
components were more regularly distributed throughout and a wavy lamellar structure was observed. In contrast, ASUM + hyalinosis samples showed distinct collagen-rich lamellae and fewer phospholipids. In ASUM − hyalinosis, phospholipids and actin accumulated in the inner part towards the lumen (Fig. 2a). The error images show that the walls are fitted well, but the deposits within the lumen of the control sample are not (Fig. S1, Supporting Information) and cannot therefore be interpreted as actin. The overlay of the distribution maps verified the co-location of the three components and the more uniform distribution in the wavy structure of the control samples was compared to the dense lamellae with accumulations in the inner and/or outer part of ASUM + and ASUM − hyalinosis (Fig. 2b).

Figure 2 Raman imaging reveals component spectra and component distribution. (a) Based on non-negative matrix factorization (NMF) analyses, three characteristic endmember spectra were derived and used as reference (basis) spectra to fit the Raman mappings of all three samples to visualize the distribution of phospholipids (basis 1, blue), collagen (basis 2, red) and actin (basis 3, yellow). (b) Combined images generated from the three components: phospholipids, collagen and actin. Measurements were taken on an arteriole of a non-hypertensive control (Control), a hyalinized arteriole (ASUM + Hyalinosis) and an arteriole without signs of hyalinosis (ASUM − Hyalinosis) from an ASUM sample. ASUM, Arteriolosclerotic ulcers of Martorell.
Distribution maps highlight differences between control and ASUM samples

Setting an intensity threshold (light blue mask in the images) on the distribution maps of phospholipids, collagen and actin allowed detailed comparison in terms of the amount and derivation of the corresponding average spectra on differences in molecular structure (Fig. 3 and Fig. S2, Supporting Information). Phospholipids were decreased in both ASUM samples and restricted to lamellae (ASUM + hyalinosis) and the inner part (ASUM − hyalinosis) (Fig. 3a). The average spectra derived from the highlighted regions were normalized to the strongest band at 2881 cm$^{-1}$ (CH stretching), which was excluded from the spectra for better comparability. The spectra showed that in addition to the typical phospholipid contributions, (1456, 1296, 1133 and 1063 cm$^{-1}$) bands from the other components (e.g. 1660, 1004, 937 and 851 cm$^{-1}$; Fig. 3b) also pointed to a co-location of components. The band typical for water at approximately 3330–3500 cm$^{-1}$ was markedly higher in the control than in ASUM + hyalinosis and ASUM − hyalinosis (Fig. 3b). Phospholipid bands (1456, 1296, 1133 and 1063 cm$^{-1}$) were reduced in both ASUM samples, 892 cm$^{-1}$ was reduced to a shoulder, and the bands at 851 and 717 cm$^{-1}$ were increased (Fig. 3b). Highlighting the collagen-rich regions resulted in different pictures of the three samples. In the control, the outermost and the innermost layers were emphasized in a wavy structure, in ASUM+, the dense lumen-sided lamellae and in ASUM−, the outermost layer were highlighted (Fig. 3c). In the derived average spectra, a decrease in the OH stretching was observed from control to ASUM− to ASUM+, together with a decrease in the lipid bands (1456, 1296, 1133 and 1063 cm$^{-1}$; Fig. 3e). In addition, these derived average spectra showed a band at 717 cm$^{-1}$, which was more pronounced in the ASUM+ sample. Finally, actin was highlighted in the outer part of the control, within the dense lamellae of ASUM+ and in the inner part of ASUM− (Fig. 3e). The derived average spectra confirmed an OH-stretching decrease in the ASUM samples and an increase of the 717 and 851 cm$^{-1}$ band. Compared to the others, these spectra included mainly protein bands, while phospholipids were the lowest within these highlighted regions. The actin bands at 620 and 640 cm$^{-1}$ were weaker than those of the pure.

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**Figure 3** Intensity thresholds on the distribution maps highlight phospholipid, collagen and actin rich areas and average spectra give molecular composition. (a–f) An intensity threshold on the distribution maps was set for better comparison of the regions rich in the three different components. Areas above the following thresholds are shown in blue: (a) phospholipids (basis 1) > 3000 and (b) the spectra derived from the highlighted areas; (c) collagen (basis 2) > 6000 and (d) average spectra; (e) actin (basis 3) > 8000 and (f) average spectra. All spectra are min-max normalized on the CH stretching (region not shown) for better comparison. Measurements were taken from an arteriole of a non-hypertensive control (Control), a hyalinized arteriole (ASUM + Hyalinosis) and an arteriole without signs of hyalinosis (ASUM − Hyalinosis) from an ASUM sample. ASUM, Arteriolosclerotic ulcers of Martorell.
endmember spectrum (Fig. 3f); thus, a mixture with other proteins was reasonable.

**Using band integration to show chemical differences**

By screening all bands, we found a peak at 937 cm$^{-1}$ (Fig. 2a) suited to visualize collagen (Fig. 4a). The peak at 1004 cm$^{-1}$ was attributed to the phenylalanine ring $^{35,37,38}$ and thus represented protein distribution. Both peaks showed similar, evenly distributed signals in the control sample. Within the hyalinated area in ASUM + hyalinosis and on the luminal side of ASUM – hyalinosis, both bands showed an intense signal within a more densely packed structure. Comparison of the amount of collagen/protein (red/pink) revealed higher protein content in the control and higher collagen content in ASUM samples. As seen in the band at 757 cm$^{-1}$, there was an evenly distributed signal in the vessel wall of the control, whereas the signal almost disappeared in ASUM + hyalinosis and ASUM – hyalinosis vessels $^{39}$ (Fig. 4a). Band integration of the peaks around 1298 and 1132 cm$^{-1}$ demonstrated the distribution of phospholipids. $^{34}$ As phospholipids are an integral component of the cell membrane, the presence of cells can be derived from their distribution. In the control group, both bands showed an evenly distributed signal with an emphasis on the luminal side. In ASUM + hyalinosis and ASUM – hyalinosis, the amount of phospholipids within the vessel wall was markedly decreased, indicating a similar decrease in cells. The band detected only in the ASUM samples at 717 cm$^{-1}$ (Fig. 3b,d,f) can be attributed to phosphatidylcholine, $^{34}$ and band integration showed a markedly stronger signal in ASUM + hyalinosis and ASUM – hyalinosis compared to the control (Fig. 4b).

**Histomorphological analysis of hyaline arteriolar changes found in renal and cutaneous biopsies of hypertensive patients**

Histological specimens from ASUM, hypertensive nephropathy and healthy skin were stained with H&E to identify hyalinized arterioles and control arterioles, respectively. In H&E staining, hyaline deposits appeared as pink areas, lacking or containing only very few nuclei (Fig. 5a). For the detection of collagen, Masson trichrome (Fig. 5b) and picrosirius red (Fig. 5c) staining were performed. In Masson-Trichrome-stained samples, hyalinized arteriolar vessel walls appear in blue at the innermost concentric structures, indicating the presence of collagen fibres within the hyaline wall of ASUM and hypertensive nephropathy.

Healthy control vessels appeared in red and blue, indicating the presence of a more profound smooth muscle component in addition to collagen layers (Fig. 3b).

These findings were supported by Picrosirius Red staining (Fig. 5c), which was analysed under polarized light. Collagen fibres appear in luminous red and yellow, which were observed in hyalinized areas of arterioles and in connective tissue (which contains abundant amounts of collagen). This effect appeared to a markedly lesser extent in the outer, non-hyalinized compartment of the vessel wall and in the media of the healthy control vessel compared to the hyalinized area (Fig. 5c).

Immunofluorescence staining for α-SMA and desmin was performed to evaluate the presence of smooth muscle cells. Both markers showed only a thin positive layer surrounding the hyalinized (unstained) inner area of the vessel wall, whereas the signal was spread evenly in healthy controls, indicating a loss of smooth muscle cells in hyalinized arterioles (Fig. 6). These findings support the RAMAN-based observations of subendothelial

**Figure 4** Band integration images for collagen, proteins, actin, phospholipids and phosphatidylcholine. (a) Typical bands for collagen (930, 1004 cm$^{-1}$) and actin (757 cm$^{-1}$) were integrated and combined. (b) Bands assigned to phospholipids (1298, 1132 cm$^{-1}$) and phosphatidylcholine (717 cm$^{-1}$) were integrated and their combination shown on the right. ASUM + Hyalinosis, hyalinized arteriole from an ASUM sample; ASUM – Hyalinosis, an arteriole without signs of hyalinosis from an ASUM sample; Control, non-hypertensive control. ASUM, Arteriolosclerotic ulcers of Martorell.
collagen-rich matrix deposits, which lack actin and therefore smooth muscle cells.

**Discussion**

Hyaline arteriolosclerosis, characterized by the hardening and loss of elasticity of the vessel wall, can be seen in several diseases associated with hypertension, including ASUM and hypertensive nephrosclerosis. Arterioles show massive thickening of the vessel wall, leaving a narrow lumen that is often occluded, entailing local ischaemic injury, which can manifest in ischaemic skin infarction, such as in ASUM, or a decline in renal function, which is evident in hypertensive nephrosclerosis.

Although several authors have previously reported on these hyaline wall changes, which can typically be found in the media of arterioles and small arteries, few attempts have been made to characterize these deposits. We introduced Raman imaging as an important tool to reveal molecular changes at the micron level within the vascular wall and hyaline deposits. Our data analysis showed that subendothelial hyaline deposits were mainly composed of collagen and phospholipids, particularly phosphatidylcholine. The markedly higher water band in the control compared to ‘ASUM + hyalinosis’ and ‘ASUM /C0 hyalinosis’ indicated a higher density of the tissue in the latter, as an indirect sign of arteriolar sclerosis present in hyaline arteriolopathy. The presence of collagen within the hyaline deposits was confirmed by Masson trichrome and picrosirius red staining. Additionally, the presence of collagen could also be shown for hypertensive nephropathy. Actin-associated Raman bands, which can be observed in arterioles of healthy non-hypertensive controls, were decreased in hyalinized vessels, corresponding to the loss of smooth muscle cells during the process of hyalinization.

The presence of collagen has previously been described in hyaline arteriolosclerosis of intertubular arteries and arterioles in the testes of mice using Masson trichrome staining. An increase in collagen type III and IV was also found in hyalinized glomeruli of patients with hypertensive nephrosclerosis through immunohistochemical staining and in situ hybridization. Our results confirmed the crucial role of collagen in hyaline arteriolosclerosis and demonstrated its presence in the media of affected arterioles of hypertensive ischaemic skin ulcers and hypertensive nephropathy. Additionally, our analysis revealed the presence of phospholipids, particularly phosphatidylcholine, and parallelly, the absence of actin in hyalinized arteriosclerotic vessels, as compared to healthy control blood vessels. This phenomenon might evolve in response to high shear stress in arterial hypertension and subsequent apoptosis of vascular smooth muscle cells (VSMCs), resulting in vascular remodelling and medial expansion with increased elastic laminal breaks, increased collagen

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synthesis and abnormal extracellular matrix deposition.\textsuperscript{43–45} VSMC apoptosis and decrease in proliferation are known to be promoted by the release of nitric oxide by endothelial cells during shear stress-induced dysfunction, which is also characterized by impaired endothelium-dependent vasorelaxation and vasodilatory response to acetylcholine.\textsuperscript{20,43,46–48} This is presumably a crucial mechanism for the pathogenesis of this disease. It has been demonstrated that patients with ASUM show higher vascular resistance compared to controls, which may lead to decreased tissue perfusion and subsequent ischaemia due to impaired compensatory vasorelaxation.\textsuperscript{49} Additionally, lysophosphatidylcholine, which is a metabolite of phosphatidylcholine and the main component of oxidatively damaged low-density lipoprotein, promotes the migration of macrophages and lymphocytes, upregulates the production of pro-inflammatory cytokines, increases oxidative stress and induces apoptosis.\textsuperscript{50,51} These mechanisms may play an important role in the pathogenesis of this disease, and (phospho-)lipid metabolism may, therefore, also be a potential therapeutic target.

In summary, the present study showed that arteriolar hyaline deposits in hypertensive arteriolopathy are predominantly composed of collagen and phospholipids, particularly phosphatidylcholine. Together with the concurrent absence of actin, these findings suggest that potentially critical disease mechanisms involve pressure-induced vascular smooth muscle cell apoptosis and subsequent collagen deposition. However, the small sample size represents a major limitation of this study and further studies are mandatory to confirm these results. These results might add substantial information that can facilitate the development of potential earlier pharmaceutical interventions even preventing these subendothelial hyaline deposits in hypertensive arteriolopathy, with the aim of preserving arteriolar function and preventing further arteriolosclerosis in affected patients.

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Supporting information
Additional Supporting Information may be found in the online version of this article:

Figure S1. Visualization of the distribution of three characteristic endmember spectra.
Figure S2. Average spectra derived from distribution maps allow detailed comparison on differences in molecular structure.