Arteriolar vascular smooth muscle cell differentiation in benign nephrosclerosis

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Abstract

Background. Benign nephrosclerosis (bN) is the most prevalent form of hypertensive damage in kidney biopsies. It is defined by early hyalinosis and later fibrosis of renal arterioles. Despite its high prevalence, very little is known about the contribution of arteriolar vascular smooth muscle cells (VSMCs) to bN. We examined classical and novel candidate markers of the normal contractile and the pro-fibrotic secretory phenotype of VSMCs in arterioles in bN.

Methods. Sixty-three renal tissue specimens with bN and eight control specimens were examined by immunohistochemistry for the contractile markers caldesmon, alpha-smooth muscle actin (alpha-SMA), JunB, smoothelin and the secretory marker S100A4 and by double stains for caldesmon or smoothelin with S100A4.

Results. Smoothelin immunostaining showed an inverse correlation with hyalinosis and fibrosis scores, while S100A4 correlated with fibrosis scores only. Neither caldesmon, alpha-SMA nor JunB correlated with hyalinosis or fibrosis scores. Cells in the arteriolar wall were exclusively positive either for caldesmon/smoothelin or S100A4.

Conclusions. This is the first systematic analysis of VSMC differentiation in bN. The results suggest that smoothelin is the most sensitive marker for the contractile phenotype and that S100A4 could be a novel marker for the secretory

Markers of αVSMC phenotype


phenotype in vivo. The other markers did not seem to differentiate these phenotypes in bN. Thus, VSMC phenotype markers should be defined in the context of the vessel segment and disease under examination. S100A4 could not only be a marker of pro-fibrotic secretory VSMCs in bN but also an important mediator of arteriolar fibrosis.

**Keywords:** arteriolosclerosis; arterionephrosclerosis; hypertensive nephrosclerosis; vascular smooth muscle cell; FSP1

### Introduction

Benign nephrosclerosis (bN), defined as kinking, hyalinosis and fibrosis of small preglomerular vessels [1], is a very common finding in renal biopsies. While considerable effort has been made to characterize the glomerular and tubulointerstitial manifestations of hypertensive renal damage; surprisingly, little is known about the changes in the affected vessels themselves.

The main constituent cell of the arterial and arteriolar wall is the vascular smooth muscle cell (VSMC). It is also functionally important for the autoregulation of glomerular blood flow and capillary pressure [2]. Smooth muscle cells have been the subject of extensive studies in diseases of the bronchi (reviewed in [3, 4]), pulmonary arteries (reviewed in [5–7]) and systemic arteries (reviewed in [8, 9]). Based on these studies and *in vitro* experiments, a paradigm of the contractile and the secretory phenotype has been developed [10, 11]. The normal physiological phenotype of VSMCs is called the contractile phenotype. The (collagen-) secretory phenotype is considered pathological. Although never formally proven in humans, the current concept of bN claims that the initial stage of bN is the hyalinotic lesion. This hyalinosis is thought to later transform into the fibrotic lesion [1]. Based on this assumption and on the paradigm above, we hypothesized that during the hyalinotic lesion, arteriolar VSMCs (aVSMCs) retain their contractile phenotype and that a transition towards the secretory phenotype S100A4, and we did double stains to explore the shift in differentiation of the aVSMCs.

### Materials and methods

**Tissue samples**

Sixty-three renal tissue samples (55 biopsies and 8 nephrectomy specimens due to laceration or tumour) with an isolated diagnosis of bN were chosen from the archive of the Institute of Pathology, Hannover Medical School, Hannover, Germany. Excluded were tissue specimens with any other primary vascular, glomerular or tubulointerstitial disease.

All biopsies were subject to routine histological workup including multiple haematoxylin and eosin-, periodic acid-Schiff (PAS)- and Jones sections, immunohistochemistry for IgA, IgG, IgM, C1q and C3c and ultrastructural examination of at least one glomerulus. These biopsies were compared to normal control kidney specimens from eight patients from tumour or trauma nephrectomy specimens without any evidence of bN.

**Clinical data**

Clinical data for the biopsies of the patients and controls were gathered from the files of the referring clinic and included age, sex, systolic and diastolic arterial blood pressure, body height, body weight, body mass index, proteinuria and serum creatinine. These clinical characteristics of the patients and control cohort are given in Table 1. The patients were significantly older than the controls and they had a higher body mass index. This mismatch could not be avoided since almost any kidney from older patients or patients with a higher body mass index that we initially considered as a normal control showed at least minimal bN.

**bN and other histologic parameters**

A semiquantitative score of bN was applied to all biopsies as recently described [12]. Arteriolar hyalinosis scores (HScores) and fibrosis scores (FScores) were given independently as 0 (absent), 1 (minimal), 2 (moderate) or 3 (severe).

In addition, the area of interstitial fibrosis and cortical tubular atrophy (IFTA) was graded in components as IFTA 0 with <10%, IFTA 1 with <25%, IFTA 2 with <50% and IFTA 3 with ≥50%.

The presence of focal segmental glomerulosclerosis (FSGS) was determined according to the definition by D’Agati *et al.* [13].

**Immunostaining**

Immunostaining was done for the smooth muscle phenotype markers given in Table 2. To this end, 3 μm sections from formalin-fixed and paraffin-embedded biopsies were stained for caldesmon, alpha-smooth muscle actin (alpha-SMA), JunB and S100A4 in fully automated Benchmark XT immunostainers (Ventana Medical Systems, Tucson, AZ) with the antigen

<table>
<thead>
<tr>
<th></th>
<th>Patients with bN (n = 63)</th>
<th>Controls without bN (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>56 ± 16.4 (n = 63)</td>
<td>22 ± 13.4 (n = 8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex (female: male)</td>
<td>17:46</td>
<td>2:6</td>
<td>0.90</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)*</td>
<td>133 ± 26.6 (n = 56)</td>
<td>109 ± 14.6 (n = 8)</td>
<td>0.0150</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)*</td>
<td>77 ± 16.2 (n = 56)</td>
<td>66 ± 8.6 (n = 8)</td>
<td>0.0349</td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>175 ± 10.4 (n = 49)</td>
<td>169 ± 24.9 (n = 7)</td>
<td>0.7848</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>83 ± 15.6 (n = 50)</td>
<td>69 ± 32.2 (n = 7)</td>
<td>0.1889</td>
</tr>
<tr>
<td>Body mass index (kg/m²)*</td>
<td>27 ± 4.8 (n = 49)</td>
<td>23 ± 5.5 (n = 7)</td>
<td>0.0386</td>
</tr>
<tr>
<td>Proteinuria*</td>
<td>25/39 (64%)</td>
<td>0/4 (0%)</td>
<td>0.0248</td>
</tr>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>170 ± 120.1 (n = 56)</td>
<td>80 ± 22.5 (n = 6)</td>
<td>0.190</td>
</tr>
</tbody>
</table>

*P < 0.05.
Markers of aVSMC phenotype

Table 2. Primary antibodies and antigen retrieval used for the characterization of aVSMCs

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Vendor, order number</th>
<th>pH/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caldesmon</td>
<td>DakoCytomation, Hamburg, Germany; M3557</td>
<td>pH 6.0 1 + 400</td>
</tr>
<tr>
<td>Alpha-SMA</td>
<td>DakoCytomation, Hamburg, Germany; M0851</td>
<td>None 1 + 25</td>
</tr>
<tr>
<td>JunB</td>
<td>Acris Antibodies GmbH, Herford, Germany; AP02568PU-N</td>
<td>pH 8.4 1 + 50</td>
</tr>
<tr>
<td>S100A4</td>
<td>Abcam plc, Cambridge, UK; ab40722</td>
<td>pH 6.0 1 + 400</td>
</tr>
<tr>
<td>Smoothelin</td>
<td>Abcam plc, Cambridge, UK; ab21108</td>
<td>pH 6.0 1 + 100</td>
</tr>
</tbody>
</table>

DakoCytomation, Hamburg, Germany; M3557

The clinical data of patients with bN and controls without bN are compared in Table 1. Patients with bN were older than controls, had higher systolic and diastolic arterial blood pressures, a higher body mass index and more frequently proteinuria. No difference in body height, body weight or serum creatinine could be found between the cohorts.

Clinical parameters in relation to histological parameters are given in Tables 3–5.

Table 3. Systolic blood pressure in relation to histological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Systolic blood pressure (mmHg)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HScore 0 (n = 11)</td>
<td>110 ± 18.6</td>
<td>0.0016</td>
</tr>
<tr>
<td>HScore 1 (n = 29)</td>
<td>125 ± 23.7</td>
<td></td>
</tr>
<tr>
<td>HScore 2 (n = 14)</td>
<td>139 ± 25.1</td>
<td></td>
</tr>
<tr>
<td>HScore 3 (n = 10)</td>
<td>152 ± 25.9</td>
<td></td>
</tr>
<tr>
<td>FScore 0 (n = 21)</td>
<td>114 ± 17.1</td>
<td>0.0023</td>
</tr>
<tr>
<td>FScore 1 (n = 22)</td>
<td>132 ± 25.3</td>
<td></td>
</tr>
<tr>
<td>FScore 2 (n = 16)</td>
<td>139 ± 29.5</td>
<td></td>
</tr>
<tr>
<td>FScore 3 (n = 5)</td>
<td>157 ± 21.2</td>
<td></td>
</tr>
</tbody>
</table>

Only patient cohort

IFTA 0 (n = 28) | 122 ± 24.5 | 0.0037  |
IFTA 1 (n = 16) | 136 ± 19.0 |         |
IFTA 2 (n = 3)  | 135 ± 39.1 |         |
IFTA 3 (n = 2)  | 160 ± 23.0 |         |
FSGS present (n = 7) | 129 ± 25.8 | 0.0073  |
FSGS absent (n = 49) | 130 ± 25.8 |         |

Table 4. Diastolic blood pressure in relation to histological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diastolic blood pressure (mmHg)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HScore 0 (n = 11)</td>
<td>67 ± 10.1</td>
<td>0.0145</td>
</tr>
<tr>
<td>HScore 1 (n = 29)</td>
<td>73 ± 14.7</td>
<td></td>
</tr>
<tr>
<td>HScore 2 (n = 14)</td>
<td>83 ± 18.2</td>
<td></td>
</tr>
<tr>
<td>HScore 3 (n = 10)</td>
<td>84 ± 14.3</td>
<td></td>
</tr>
<tr>
<td>FScore 0 (n = 21)</td>
<td>125 ± 23.7</td>
<td>0.0220</td>
</tr>
<tr>
<td>FScore 1 (n = 22)</td>
<td>77 ± 11.6</td>
<td></td>
</tr>
<tr>
<td>FScore 2 (n = 16)</td>
<td>79 ± 22.3</td>
<td></td>
</tr>
<tr>
<td>FScore 3 (n = 5)</td>
<td>88 ± 16.8</td>
<td></td>
</tr>
</tbody>
</table>

Only patient cohort

IFTA 0 (n = 28) | 72 ± 15.7 | 0.0015  |
IFTA 1 (n = 16) | 78 ± 15.1 |         |
IFTA 2 (n = 3)  | 70 ± 10.0 |         |
IFTA 3 (n = 9)  | 93 ± 11.3 |         |
FSGS present (n = 7) | 75 ± 16.2 | 0.0181  |
FSGS absent (n = 49) | 89 ± 10.3 |         |

Systolic blood pressure correlated with HScores (P = 0.0016) and FScores (P = 0.0023). After exclusion of the control cohort, it correlated with IFTA scores (P = 0.0037) and the presence of FSGS (P = 0.0070).

Diastolic blood pressures correlated also with HScores (P = 0.0145) and FScores (P = 0.0220). When analysing only the patient cohort, a correlation was found with IFTA scores (P = 0.0015) and the presence of FSGS (P = 0.0181).

Patients’ serum creatinine correlated with HScores (P = 0.0013), FScores (P = 0.0015) and with IFTA scores (P = 0.0010). No significant correlation was found with the presence of FSGS.

Results

Clinical parameters in patients and controls

The clinical data of patients with bN and controls without bN are compared in Table 1. Patients with bN were older than controls, had higher systolic and diastolic arterial blood pressures, a higher body mass index and more frequently proteinuria. No difference in body height, body weight or serum creatinine could be found between the cohorts.

Clinical parameters relative to histological parameters

Clinical parameters in relation to histological parameters are given in Tables 3–5.

Statistical analysis

All statistical analyses were carried out with JMP 8.0.2 (SAS Institute, Cary, NC) on an Apple Macintosh (Apple, Cupertino, CA). For comparison of nominal parameters, chi-square tests were used and for continuous parameters, Wilcoxon tests. Differences were regarded as significant with P < 0.05 in two-sided tests. However, in this retrospective exploratory study, P-values can only be regarded as descriptive.

Ethical approval

All studies were carried out according to the Declaration of Helsinki in its latest revision [14] and were approved by the ethics committee of Hannover Medical School.
Correlations between glomerular, vascular and tubulointerstitial histological parameters

As shown in Figure 1, the IFTA scores correlated with HScores (P < 0.0001) and FScores (P = 0.0001).

No significant correlation was found between HScores or FScores and the presence of FSGS.

Markers of aVSMC differentiation

Caldesmon immunostaining was found only in the arteriolar wall in a diffuse cytoplasmic pattern (see Figure 2D–F). The staining intensity in relation to HScores and FScores is given in Figure 3A and B. No significant correlation was found between arteriolar wall immunostaining and HScores (kappa = −0.052235, P = 0.0558) and FScores (kappa = −0.03323, P = 0.0812).

Alpha-SMA immunostaining was found, similar to that of caldesmon, to be diffusely cytoplasmic (see Figure 2G–I). No significant correlation with HScores (kappa = −0.065, P = 0.515) and FScores (kappa = 0.02289, P = 0.5055) was found (see Figure 3C and D).

Smoothelin immunostaining was present in a diffuse cytoplasmic pattern (see Figure 2J–L). As shown in Figure 3E and F, staining intensity was significantly decreased with even minimal HScores (kappa = −0.11552, P < 0.0001) and FScores (kappa = −0.18724, P = 0.0033).

JunB was found in a predominantly nuclear staining pattern in the arteriolar wall (see Figure 2M–O). No significant correlation between arteriolar wall immunostaining of FSP-1 (kappa = 0.29318, P = 0.0321; see Figure 3I and J).

Arteriolar endothelium was found to be consistently positive for S100A4.

Double immunofluorescence stainings of markers of aVSMC differentiation

Representative examples of normal control arterioles and arterioles with hyalinotic and fibrotic lesions were concordant with the single marker immunoperoxidase staining. The combination of caldesmon and S100A4 showed that aVSMCs were either positive for caldesmon or S100A4. No transitional shift in differentiation with loss of caldesmon and gain of S100A4 could be demonstrated (see Figures 4–6). The same mutually exclusive staining pattern was found in the double stains for smoothelin and S100A4: cells in the arteriolar wall were either completely positive for smoothelin in the normal arterioles (Figure 7) or for S100A4 in the hyalinotic lesions (Figure 8) and in the fibrotic lesions (Figure 9).

Markers of aVSMC differentiation relative to clinical parameters

None of the markers caldesmon, alpha-SMA, smoothelin, JunB or FSP-1 correlated with systolic blood pressure, diastolic blood pressure or serum creatinine (data not shown).

Table 5. Serum creatinine in relation to histological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum creatinine (μmol/L)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HScore 0 (n = 8)</td>
<td>88 ± 35.7</td>
<td>0.0013</td>
</tr>
<tr>
<td>HScore 1 (n = 27)</td>
<td>134 ± 77.9</td>
<td></td>
</tr>
<tr>
<td>HScore 2 (n = 16)</td>
<td>153 ± 103.4</td>
<td></td>
</tr>
<tr>
<td>HScore 3 (n = 11)</td>
<td>294 ± 159.4</td>
<td></td>
</tr>
<tr>
<td>FScore 0 (n = 20)</td>
<td>106 ± 43.2</td>
<td>0.0105</td>
</tr>
<tr>
<td>FScore 1 (n = 20)</td>
<td>151 ± 95.3</td>
<td></td>
</tr>
<tr>
<td>FScore 2 (n = 17)</td>
<td>177 ± 124.5</td>
<td></td>
</tr>
<tr>
<td>FScore 3 (n = 5)</td>
<td>370 ± 158</td>
<td></td>
</tr>
<tr>
<td>Only patient cohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFTA 0 (n = 27)</td>
<td>117 ± 57.8</td>
<td>0.0010</td>
</tr>
<tr>
<td>IFTA 1 (n = 16)</td>
<td>148 ± 54.8</td>
<td></td>
</tr>
<tr>
<td>IFTA 2 (n = 4)</td>
<td>150 ± 58.2</td>
<td></td>
</tr>
<tr>
<td>IFTA 3 (n = 9)</td>
<td>376 ± 152.2</td>
<td></td>
</tr>
<tr>
<td>FSGS present (n = 7)</td>
<td>164 ± 115.1</td>
<td>0.5520</td>
</tr>
<tr>
<td>FSGS absent (n = 49)</td>
<td>212 ± 154.4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. IFTA scores in relation to HScores and FScores after exclusion of the controls. Both HScore and FScore showed a significant correlation with IFTA scores (P < 0.05).
Among the classical markers of VSMC differentiation, only the contractile marker smoothelin correlated with arteriolar hyalinosis and fibrosis as expressed by the HScores and FScores in bN. The other examined classical markers of the contractile phenotype caldesmon and alpha-SMA did not. Furthermore, the transcription factor JunB, which regulates the arterial contractile capacity [15] and thus appeared as a potential novel marker of the contractile phenotype, correlated with neither HScores nor FScores. Does this mean that the paradigm of VSMC phenotype is not valid for renal parenchymal aVSMCs in bN? In our opinion, this valuable paradigm should not be abandoned based merely on the results in the special setting of bN. In our opinion, the results just argue for a redefinition and interpretation of the differentiation markers in the context of the type of vessel and disease examined. The notion that caldesmon is a marker of the contractile phenotype stems from examinations in the fetal and adult aorta [16, 17] and from cell culture experiments [18]. The same is true for alpha-SMA [16–19]. Considering the diversity of VSMCs along the course of the vascular tree [20], it appears logical that the markers derived from studies of the aorta do not necessarily apply to renal parenchymal arterioles. Still, smoothelin seems to be a universal marker of VSMC differentiation at all levels of the arterial bed: studies on hypertensive remodeling of the mini-pig aorta have shown a decrease in smoothelin expression [21] quite similar to that in the renal parenchymal arterioles in the present study.

In this study, we also examined the transcription factor JunB in aVSMCs. JunB is a transcription factor that regulates actinomysin-based cell contraction via the expression of myosin light chain 9 (My9). Deletion of JunB has been shown to strongly reduce the contraction capability of arteries in vivo [15]. Preserved nuclear JunB immunostaining even in kidneys with higher scores of hyalinosis and fibrosis suggests that JunB-dependent regulation of aVSMC contraction is not disturbed in bN. However, this does not imply that the contraction capacity of arterioles in bN is intact since it also depends on other factors such as arteriolar diameter, cellular and matrix composition of the vessel wall and the local concentration of vasoactive mediators. Neither does the lack of evidence for decreased nuclear JunB immunostaining in VSMCs of hyalinotic or fibrotic arterioles exclude JunB as a marker of the contractile phenotype in VSMCs of other vascular segments and in other disease settings.

While there are many markers of the contractile phenotype of VSMCs, there is currently no widely accepted positive defining marker of the in vivo secretory phenotype [22]. Based on in vitro data about spindled (contractile) and rhomboid (secretory) VSMCs [23], we speculated that the ‘rhomboid’ marker S100A4, also known as p9Ka, MTS1 or fibroblast-specific protein 1 (FSP1), might serve as a marker of the secretory phenotype in bN. The name FSP1 has been given to S100A4 because of its prominent role in kidney fibrosis [24]. S100A4 was originally thought to be expressed almost exclusively by fibroblasts [24]. Later studies have found S100A4 expression in mononuclear cells, questioning its specificity for fibroblasts [25, 26]. Still S100A4 is a
Fig. 3. αVSMC differentiation markers in relation to HScores and FScores. Smoothelin immunostaining of αVSMCs correlated with HScores (kappa = −0.11552; P < 0.0001) and FScores (kappa = −0.18724; P = 0.0033). S100A4 immunostaining correlated with FScores only (kappa = 0.29318; P = 0.0321).
Fig. 4. Distribution of caldesmon (red) and S100A4 (green) in a normal arteriole. The top left panel shows the appearance on the Gill’s haematoxylin-stained section with bright-field microscopy for exact morphological reference, while the top right and the bottom left panels show the immunofluorescence staining patterns of caldesmon (red) and S100A4 (green). The bottom right panel shows the merged immunofluorescence staining. Note that all cells in the arteriolar wall are caldesmon positive and S100A4 negative. Only the endothelial lining is positive for S100A4 in this normal arteriole. Combined Gill’s haematoxylin stain with caldesmon and S100A4 double immunofluorescence staining on the same section, original magnification ×600.

Fig. 5. Double immunofluorescence staining of caldesmon (red) and S100A4 (green) of a hyalinotic arteriole. The top left panel with the Gill’s stain shows hyalnosis (black arrow) and the top right and the bottom left panels show the immunofluorescence staining patterns of caldesmon (red) and S100A4 (green). The bottom right panel shows the merged immunofluorescence staining. Note the hyalnosis (black arrow) on the Gill’s stain, the S100A4-positive endothelial cells (white arrow) in the bottom left frame and the caldesmon-positive smooth muscle cells in the top right frame. Combined Gill’s haematoxylin stain, with caldesmon and S100A4 double immunofluorescence staining on the same section, original magnification ×600.

Fig. 6. Double immunofluorescence staining of caldesmon (red) and S100A4 (green) of a fibrotic arteriole. The arteriole with fibrosis is shown in a Gill’s haematoxylin stain in the top left frame, the top right and the bottom left frames show the immunofluorescence staining patterns of caldesmon (red) and S100A4 (green). The bottom right panel shows the merged immunofluorescence stain. Note the single S100A4-positive cell (arrow) that is negative for caldesmon. It is situated in the media, just outside of the violet autofluorescent lamina elastica interna. The other smooth muscle cells in the media are caldesmon positive and S100A4 negative. Combined Gill’s haematoxylin, caldesmon and S100A4 double immunofluorescence stain on the same section, original magnification ×600.

Fig. 7. Distribution of smoothelin (red) and S100A4 (green) in a normal arteriole. The Gill’s haematoxylin stain is displayed in the top left frame; the bottom left frame shows the S100A4 immunofluorescence stain in green and the top right frame shows the smoothelin stain in red. The merged frame with smoothelin and S100AB is found at bottom right. Note S100A4-positive endothelial lining and the smoothelin-positive S100A4-negative aVSMCs. Combined Gill’s haematoxylin, smoothelin and S100A4 double immunofluorescence stain on the same section, original magnification ×600.
central protein in the concept of epithelial–mesenchymal transdifferentiation, a process that transforms tubular epithelial cells into fibroblast-like cells, thus contributing to kidney fibrosis. This concept of epithelial–mesenchymal transdifferentiation has recently been seriously challenged [27]. S100A4 is a member of the S100 protein family, all of which share the calcium-binding EF-hand motif [28]. S100A4 forms dimers which bind other proteins, thus allowing cross-bridging for these proteins. A known binding partner of S100A4 is methionine aminopeptidase 2 (MetAP2) [29]. Studies in lung fibrosis have suggested a pharmacologically inhibitable role for MetAP2 in fibroblast and myofibroblast proliferation and collagen deposition [30]. In addition to its intracellular functions, S100A4 is also secreted by VSMCs [31]. The secretion of S100A4 by fibroblasts has been shown to be stimulated by CCL5 [32]. CCL5 in turn is known to be induced by injury in murine arterial VSMCs [33]. In vitro data have shown that secreted S100A4 stimulates fibronectin production in fibroblasts [32]. Fibronectin is long known to play an important role in arterial fibrosis (reviewed in [34]). Secreted S100A4 can induce osteopontin in an osteosarcoma cell line [35]. Osteopontin is a major effector of hypertensive arterial fibrosis in rats [36]. Taken together, these literature data and the correlation of S100A4 immunostaining with FScores but not HScores suggest that S100A4 is not only a robust marker of the secretory phenotype in bN but could also have central role in fibrotic remodelling of arteriolar walls in bN.

The double immunofluorescence stainings of caldesmon and smoothelin in combination with S100A4 suggest how the arteriolar walls in bN are remodelled. No coexpression of the contractile markers caldesmon or smoothelin with S100A4 was found. Mutually exclusive expression of the differentiation markers smoothelin and S100A4 is not surprising since both markers probably represent the opposite ends of the aVSMC differentiation. However, the lack of coexpression of S100A4 even with the ‘broader’ contractile marker caldesmon, as shown in Figure 6, suggests that the transdifferentiation, from contractile to secretory phenotype in the special setting of arterioles affected by bN, is probably not a gradual process but rather an abrupt switch of differentiation. Future research with more combination stainings of selected markers will be necessary to delineate differentiation patterns and pathways in the fibrotic remodelling of arterioles in bN.

In summary, the present study provides the first systematic analysis of classical markers of the contractile and the secretory aVSMC phenotype in bN. The only reliable markers of the contractile phenotype in the setting of bN seem to be the recently established markers ADAMTS13 and smoothelin [12], whereas caldesmon and alpha-SMA immunostaining are stable even in severe hyalinitic and fibrotic arteriolar lesions and thus appear less suitable as a phenotypic marker in bN. In addition, the present results and literature data suggest that S100A4 is not only a robust marker of the secretory phenotype but could also be involved in the pathogenesis of fibrotic lesions in bN. The mechanisms of how S100A4 could orchestrate fibrotic remodelling in bN should be examined in future studies.

Fig. 8. Distribution of smoothelin (red) and S100A4 (green) in a hyalinotic arteriole. The top left frame shows the Gill’s haematoxylin stain, the bottom left frame is the S100A4 immunofluorescence stain in green and the top right frame the smoothelin stain in red. The merged frame with smoothelin and S100AB is shown at bottom right. No smoothelin-positive cells are present in the arteriolar wall. Note the S100A4-positive endothelial cells (arrow). All cells in the hyalinotic media are positive only for S100A4. Combined Gill’s haematoxylin, smoothelin and S100A4 double immunofluorescence staining on the same section, original magnification ×600.

Fig. 9. Distribution of smoothelin (red) and S100A4 (green) in a fibrotic arteriole. The top left frame is the Gill’s haematoxylin stain, the bottom left frame displays the S100A4 immunofluorescence stain in green and the top right frame shows the smoothelin stain in red. The merged frame with smoothelin and S100AB is shown at bottom right. No smoothelin-positive cells are present in the arteriolar wall. Note the S100A4-positive endothelial cells (arrow). All cells in the fibrotic wall are positive only for S100A4 and negative for smoothelin. Combined Gill’s haematoxylin, smoothelin and S100A4 double immunofluorescence staining on the same section, original magnification ×600.
Markers of aVSMP phenotype

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