# Proliferating cells in HIV and pamidronate-associated collapsing focal segmental glomerulosclerosis are parietal epithelial cells

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**Collapsing focal segmental glomerulosclerosis (cFSGS) is characterized by hyperplasia of glomerular epithelial cells. In a mouse model of FSGS and in a patient with recurrent idiopathic FSGS, we identified the proliferating cells as parietal epithelial cells (PECs). In the present study, we have evaluated the origin of the proliferating cells in cFSGS associated with human immunodeficiency virus (HIV) and pamidronate. We performed a detailed study of glomerular lesions in biopsies of two patients with HIV-associated cFSGS and a nephrectomy specimen of a patient with pamidronate-associated cFSGS. Glomeruli were studied by serial sectioning using light and electron microscopy and immunohistochemistry to determine the epithelial cell phenotype. We used Synaptopodin, vascular endothelial growth factor, and CD10 as podocyte markers, CK8 and PAX2 as PEC markers and Ki-67 as marker of cell proliferation. The newly deposited extracellular matrix was characterized using antiheparan sulfate single-chain antibodies. The proliferating cells were negative for the podocyte markers, but stained positive for the PEC markers and the cell proliferation marker Ki-67. The proliferating PAX-2 and CK8 positive cells that covered the capillary tuft were always in continuity with PAX-2/CK8 positive cells lining Bowman's capsule. The matrix deposited by these proliferating cells stained identically to Bowman's capsule. Our study demonstrates that PECs proliferate in HIV and pamidronate-associated cFSGS. Our data do not support the concept of the proliferating, dedifferentiated podocyte.**

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Focal segmental glomerulosclerosis (FSGS) is one of the most common patterns of glomerular injury.[1] Recently, various morphological variants have been described and a new classification was proposed.[2] The traditional forms of FSGS now classified as perihilar and FSGS – not otherwise specified, lack epithelial cell proliferation and are characterized by mesangial sclerosis, obliteration of glomerular capillaries, formation of adhesions between the glomerular tuft and Bowman's capsule, podocyte hypertrophy, hyalinosis, and intracapillary foam cells. In contrast, epithelial cell proliferation is prominent in the other variants, in particular in collapsing FSGS, defined by segmental or global collapse of the glomerular tuft associated with epithelial cell hypertrophy and hyperplasia.

In recent studies on the pathogenesis of FSGS, a central role was assigned to the visceral epithelial cell, the so-called podocyte. The podocyte is considered to be a highly specialized and differentiated cell, unable to replicate. Kriz *et al.*[3–5] have studied the development of FSGS in various rat model. Based on these studies, Kriz and Mundel (reviewed in Mundel and Shankland[6]) proposed that podocyte loss (podocytopenia) was the critical starting event in FSGS. Neighboring podocytes cannot compensate for this loss, resulting in denudation of the glomerular basement membrane and formation of an adhesion between the capillary tuft and the parietal epithelial cells (PECs).

The concept of the terminally differentiated, not-replicating podocyte seemed incompatible with the development of the proliferative lesions observed in collapsing FSGS. Since the proliferating cells cover the glomerular tuft they are considered by several investigators to be of podocytic origin,[7,8] although they do not express podocyte markers such as synaptopodin, vascular endothelial growth factor (VEGF) and Wilm's tumor antigen. These and similar observations have fostered the concept that proliferating, dedifferentiated podocytes are the main cell type involved in the formation of cellular lesions in collapsing FSGS.[9–11] Few studies have questioned this concept, apparently because of the localization of the cells, despite the observation that the

proliferating cells express cytokeratins, a marker expressed by PECs and not by podocytes.[12–14]

We previously studied the origin of the proliferating epithelial cells in FSGS in more detail in the Thy-1.1 transgenic mouse (a model of collapsing FSGS), providing evidence that in this model the proliferating epithelial cells are of PEC origin.[15] Recently, our conclusions were confirmed and strengthened by Asano *et al.*,[16] who found PEC proliferation in their transgenic mouse with LacZ expressing podocytes. Subsequently, we performed detailed studies of glomerular lesions in a patient with recurrent idiopathic FSGS.[17] Using three-dimensional analysis we were able to firmly establish that the proliferating cells were of PEC origin.

Thus, our previous studies questioned a major contribution of proliferating podocytes to the formation of cellular lesions in FSGS in general and we discussed the possibility that in this respect human immunodeficiency virus (HIV) associated collapsing FSGS (cFSGS) may be an exception.[17] In the present study, we have investigated the characteristics of the proliferating epithelial cells in glomerular lesions of patients with HIV and pamidronate-associated FSGS. Our study indicates that also in these conditions, most proliferating cells are PECs.

## RESULTS

We studied biopsies from two HIV patients with collapsing FSGS and a nephrectomy specimen from a patient with pamidronate-associated collapsing FSGS. The latter patient was treated with aminohydroxypropylidene bisphosphonate (APD: 'pamidronate') because of persistent hypercalcaemia after renal transplantation; she received oral APD in dayly dosage of 300 mg for 20 months, 450 mg for 6 months and 600 mg for 2 months. Subsequently, intravenous (i.v.) APD was administered in a dose of 60 mg at two-weekly intervals. Severe proteinuria was noted after the third infusion.

The HIV biopsies contained 11 and 17 different glomeruli, respectively, and the paraffin block from the nephrectomy specimen contained over 77 glomeruli. The material was serially sectioned and from each specimen more than 100 sections were examined. One of every five sections was periodic acid Schiff stain stained for light microscopy (LM) evaluation and intervening sections were retained for immunostainings.

In the biopsies with HIV-associated nephropathy (HIVAN), we observed a wide spectrum of glomerular lesions by LM. At the one end of the spectrum, there were normal appearing glomeruli and at the other end glomeruli with advanced sclerosis. For our analyses we focussed on glomeruli with 'early' collapsing lesions, as defined by collapse of the glomerular tuft without, or with only minimal, sclerosis (deposition of new matrix). In these glomeruli, the collapsed tuft was either still covered with a single layer of epithelial cells with only limited segmental proliferation (two out of 28) or collapse was accompanied by a prominent increase of epithelial cells, filling Bowman's space (18 out of 28). These

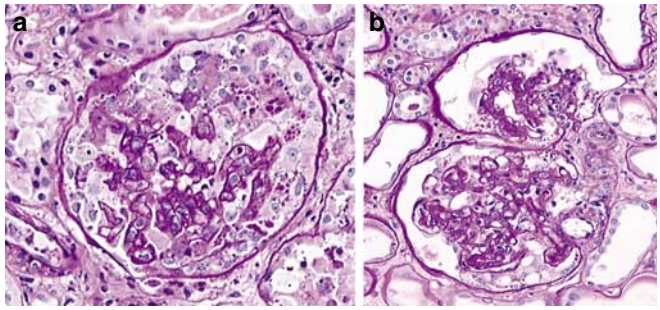


**Figure 1 | Glomerular histology in HIVAN and pamidronate nephropathy**. Glomerular histology (periodic acid Schiff stain staining) in (**a**) HIV-associated cFSGS and (**b**) pamidronate-associated cFSGS. (**a**) (HIVAN) shows a glomerulus with collapse of the capillary tuft and prominent epithelial cell proliferation in Bowmans space. Most of the glomeruli in our HIV biopsies had this appearance (18 out of 28). The epithelial cells appeared activated, with enlarged nuclei with prominent nucleoli and vacuolation of the cytoplasm with the presence of reabsorption droplets. (**b**) Podocyte injury with enlargement, vacuolation and ballooning was more evident in pamidronate-associated cFSGS and multilayering of epithelial cells in Bowman's space was only sporadically and segmentally observed (**a**, **b** $\times$ 450).

epithelial cells appeared activated, with enlarged nuclei with prominent nucleoli and vacuolation of the cytoplasm with the presence of reabsorption droplets (Figure 1a). Some glomeruli showed advanced sclerosis (seven out of 28) and one glomerulus appeared normal. True adhesions of the tuft to Bowman's capsule were not found when analyzing serial sections. Ultrastructural analysis by transmission electron microscopy showed the presence of tubular reticular inclusions in the glomerular endothelial cells of both HIV biopsies. Podocytes appeared activated and showed foot process effacement and microvillous transformation. The podocytes in pamidronate nephropathy were enlarged and highly vacuolated. The morpholocial appearance of collapsing lesions in pamidronate-associated cFSGS differed from those in HIVAN, as in this condition we usually observed only a single layer of epithelial cells in Bowman's space, covering the glomerular tuft (34 out of 77). In five of 77 glomeruli, we observed segmental multilayering of epithelial cells in Bowman's space. It was noted that these cells on top of the tuft appeared severely injured with extreme vacuolation and ballooning, more so than in HIVAN (Figure 1b). Also, in pamidronate-associated cFSGS adhesions were not found.

For phenotypic analysis of the epithelial cells in Bowman's space serial sections were stained with podocyte markers (synaptopodin, CD10, and VEGF), PEC markers (CK8 and paired cox protein 2 (PAX-2)) and proliferation marker Ki-67. To characterize the extracellular matrix we stained with an anti-heparan sulfate (HS) single-chain antibody that in normal glomeruli predominantly stains Bowman's capsule and not the glomerular basement membrane (HS4E4) and one that stains only the glomerular basement membrane and the mesangial matrix (HS4C3) (markers are described in Table 1). To strengthen our findings, we additionally

**Table 1 | Antibodies used for the detection of glomerular antigens**

| Antigen | Primary antibody | Dil. | Supplier/reference |
|---|---|---|---|
| *Podocyte components* | | | |
| Synaptopodin | Mouse anti-synaptopodin | 1 | Progen, Heidelberg, Germany |
| CD10 | Rabbit anti-human CD10 | 50 | Monosan, Caltag lab., Burlinggame |
| VEGF | ISH m-RNA Probe (*ng/ml) | 100* | [26] |
| *PEC components* | | | |
| PAX-2 | Rabbit anti-human PAX-2 | 50 | Zymed laboratories Inc. San Francisco, USA |
| Cytokeratin 8 | Mouse anti-human CAM 5.2 (CK8) | 50 | BD Biosciences, San Diego, CA |
| *Proliferation* | | | |
| Ki-67 | Mouse anti-human MIB-1 | 200 | Dako, Glostrup, Denmark |
| *Bouwman's capsule* | | | |
| Heparan sulfate single chain | Single-chain antibody HS4E4 | 1 | [25] |
| *GBM* | | | |
| Heparan sulfate single chain | Single-chain antibody HS4C3 | 1 | [24] |

GBM, glomerular basement membrane; PEC, parietal epithelial cell; VEGF, vascular endothelial growth factor.
*Concentration=Dil. 100 ng/ml.

performed VEGF/CK8, Synaptopodin/CK8 and CD10/CK8 double stainings.

In normal control kidney tissue, the cells lining Bowman's capsule (PEC) uniformly stained for PAX-2 and were negative (occasional weak positivity was observed) for CK8 (not shown). Podocytes uniformly stain for synaptopodin and CD10. CD10/CALLA is not a podocyte specific marker but in humans strongly stains podocytes in addition to brush border and vascular smooth muscle cells.[18] In HIVAN normal looking glomeruli also demonstrated uniform PAX-2 staining and additionally showed strong CK8 staining in a segmental pattern, compatible with activation of PECs (Figure 2a). In glomeruli with collapse and limited cell proliferation, CK8 positive cells segmentally covered the capillary tuft (Figure 2c and d). These cells were always in contact with CK8-positive cells lining Bowman's capsule. The remainder of the tuft was covered with a single layer of cells that were negative for all PEC and podocyte markers except for CD10, which was strongly and uniformly positive. In glomeruli with prominent hypercellularity epithelial proliferation was confimed by positive staining for Ki-67 (Figure 3b). Most cells in Bowman's space were CK8 and PAX 2 positive (Figure 3a). In these glomeruli, there was often no or only faint expression of synaptopodin (Figure 3c). Notably, most cells in the periphery of the glomerular tuft were positive for CD10 (Figure 3d), thus confirming that podocytes were still present. To strengthen these findings, we additionally performed Synaptopodin/CK8 (Figure 3e) and CD10/CK8 (Figure 3f) double stainings. No cells were found doubly positive for synaptopodin/CD10 or CK8. In more advanced lesions eventually the podocytes lost all there markers.

Also in pamidronate nephropathy we observed focal and segmental CK8 expression in normal appearing glomeruli (Figure 4a, left). In glomeruli, with collapse CK8 positive cells were found overlying the capillary tuft (Figure 4a, right) and occasionally continuity of CK8 positive cells covering the tuft

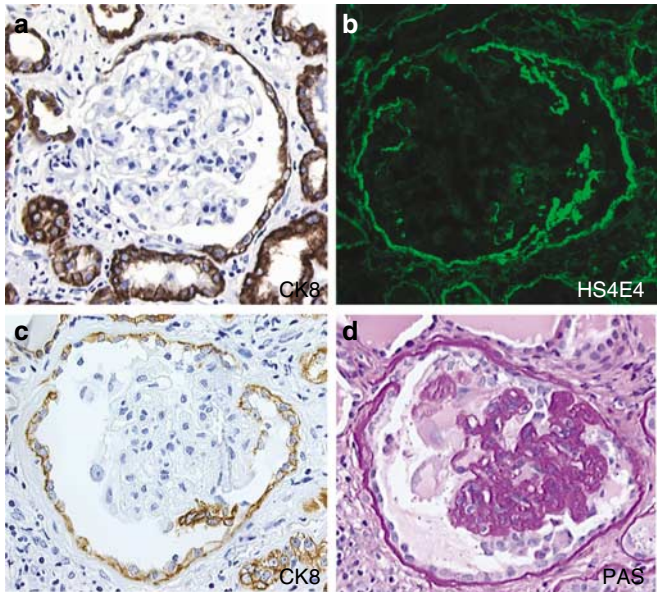


**Figure 2 | Phenotype of proliferating epithelial cells in HIV-associated nephropathy in segmental lesions.** (**a**) A section of a normal looking glomerulus (no lesions) with segmental CK8 positive cells lining Bowman's capsule. (**b**) The anti-HS single-chain antibody HS4E4 showed that newly deposited matrix in areas of epithelial proliferation stained as Bowman's capsule, shown for a glomerulus with more advanced sclerosis. (**c** and **d**) In glomeruli with collapse, podocyte hypertrophy and limited cell proliferation, CK8 positive cells segmentally covered the capillary tuft. These cells were always in contact with CK8 positive cells lining Bowman's capsule, as demonstrated in serial sections (**a**–**d** $\times$ 450).

and CK8 positive cells covering Bowman's capsule was seen away from the vascular pole. VEGF expression is present in a normal appearing glomerulus (Figure 4a, left) and is absent in an affected glomerulus (Figure 4a, right). The expression pattern observed in the normal appearing glomerulus is identical to the expression observed in normal glomeruli in

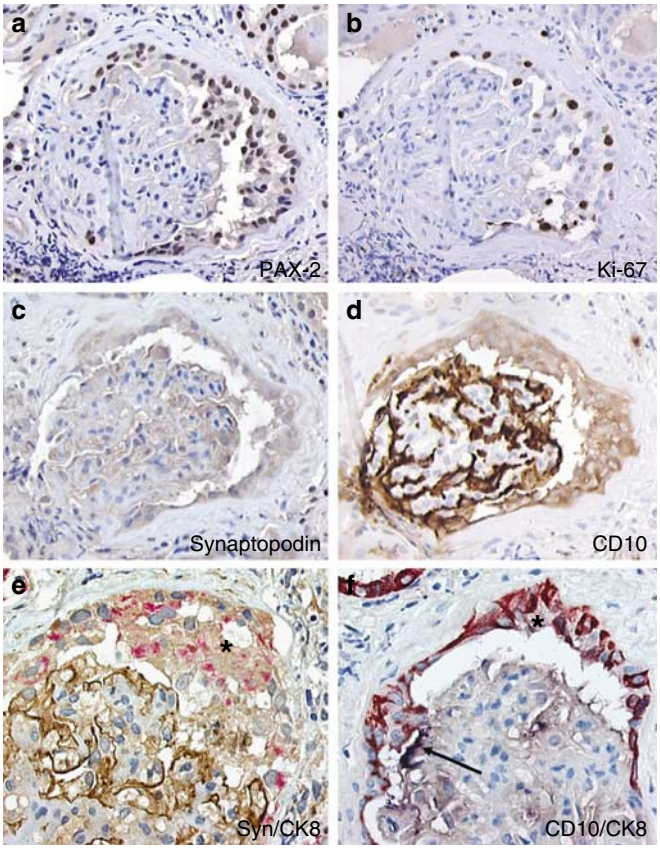


**Figure 3 | Phenotype of proliferating epithelial cells in HIV-associated nephropathy in glomeruli with extensive proliferation.** In (**a**–**d**) serial sections are shown. The proliferating cells were mostly CK8 positive (**e**, **f** – red staining) and almost all were PAX 2 positive (**a**). (**b**) Proliferation was confimed by Ki-67. (**c**) Dedifferentiated podocytes lost expression of synaptopodin, (**d**) but were still positive for CD10. Doublestainings (**e**) Synaptopodin/CK8 and (**f**) CD10/CK8 confirmed that the proliferating cells (asterisk) were positive for CK8 and are found adjacent/on top of Synaptopodin or CD10 positive residual podocytes. No cells were found doubly positive for Synaptopodin/CD10 and CK8. In more advanced lesions, podocytes lost all there markers. In (**f**) (arrow), a single podocyte has kept its expression for CD10 (**a**–**d** $\times$450, **e**, **f** $\times$700).

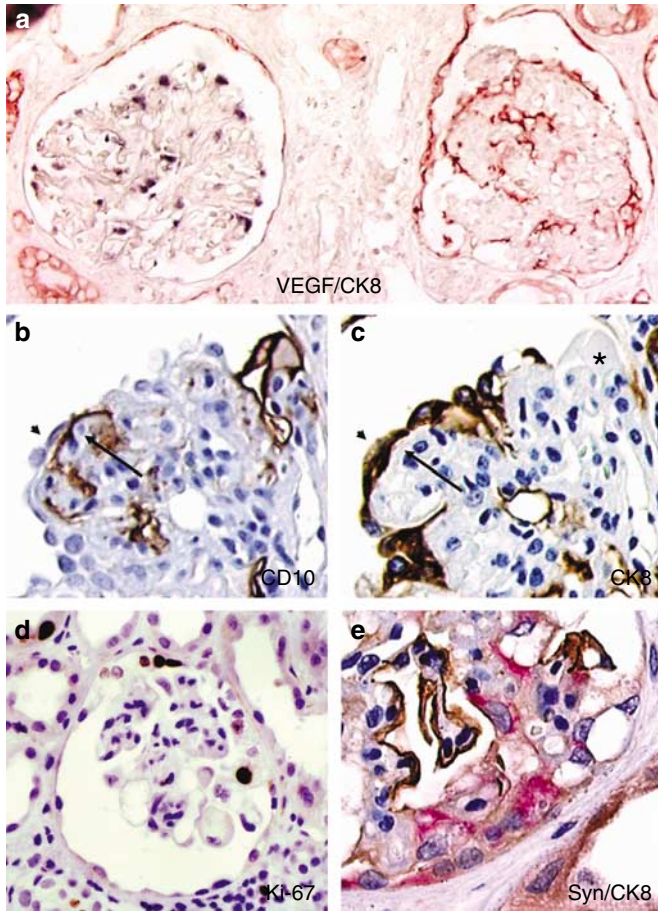


**Figure 4 | Phenotype of epithelial cells in pamidronate nephropathy.** We observed a focal and segmental CK8 expression by PECs lining Bowmans capsule, in normal appearing glomeruli (**a**, left, red staining). (**a**, right) In more advanced glomerular lesions, CK8 positive cells were also found overlying the capillary tuft. VEGF expression is (**a**, left, blue staining) present in podocytes of a normal appearing glomerulus and (**a,** right) is absent in an affected glomerulus. In serial sections, (**b**, arrow) segmental CD10 positivity was observed, (**b**, arrowhead) underneath CK8 positive cells, indicating the presence of some residual podocytes. Sometimes CK8 positive cells covering the tuft were interspersed with CK8 negative but CD10 positive cells. These CD10 positive cells showed already vacuolization and ballooning (**b**, **c**, asterisk). This latter finding was also confirmed by a Synaptopodin/CK8 double staining (**e**). We never observed VEGF/CK8 coexpression or Synaptopodin/CK8 coexpression. Epithelial cells lining Bowman's capsule and the glomerular tuft were mitotically active (**d**) althought proliferation in HIVAN was more extensive (**a**, **d** $\times$450, **b**, **c**, **e** $\times$900).

humans or mice (see Supplementary Figure S1). Ki-67 positivity was noted in PEC's covering Bowman's capsule (Figure 4d). Segmentally CD10 positivity was observed underneath CK8 positive cells (Figure 4b and c). Of note, CK8 positive cells covering the tuft were interspersed with CK8 negative CD10 positive cells, indicating the presence of residual podocytes (Figure 4c, asterisk). This latter finding was also confirmed by a Synaptopodin/CK8 (Figure 4e) double staining. We never observed coexpression of CK8/PAX2 with podocytemarkers!

In both HIV and Pamidronate-associated cFSGS staining with the anti-HS single-chain antibody HS4E4 showed that new matrix that stained as Bowman's capsule was sometimes deposited in areas of epithelial proliferation. More extensive deposition of HS4E4 positive extracellular matrix was seen in glomeruli with more advanced sclerosis (Figure 2b).

## DISCUSSION

Our study demonstrates that most of the proliferating cells in HIV and pamidronate-associated collapsing FSGS are PECs. This conclusion is based on the finding that the proliferating cells are often positive for CK8, always positive for the PEC marker PAX2 and negative for the podocyte markers synaptopodin, CD10, and VEGF. In addition, the staining characteristics of the extracellular matrix that is produced by the proliferating cells are identical to those of Bowman's capsule. Admittedly, the use of cellular markers to define the origin of proliferating cells can be questioned, since it can be

argued that cellular activation may induce phenotypic changes and could cause a switch from an activated podocyte phenotype to a parietal cel phenotype. However, the CK8 and PAX-2 positivity strongly supports the PEC origin of these cells as shown in our previous study.[17] In normal kidney, PECs are negative or stain only faintly positive for CK8. In some of the normal appearing glomeruli in our patients, there was strong CK8 staining of cells lining Bowman's capsule in a segmental distribution. By serial sectioning, we could show that in these glomeruli adhesions between the glomerular tuft and Bowman's capsule were absent, thus providing a strong argument that these PECs acquire the CK8 positive phenotype upon activation, prior to proliferation. Perhaps, the most striking argument for our conclusion is the demonstration of CK8 positive cells on top of residual CD10 and even Synaptopodin positive podocytes by double staining.

Our present findings in HIV and pamidronate-associated cFSGS confirm and extend the conclusions of our previous studies. In the Thy-1.1 transgenic mouse, a model of collapsing FSGS, we observed that the proliferating epithelial cells strongly express PEC markers. In addition, we noted that the staining characteristics of the newly formed extracellular matrix are identical to Bowman's capsule arguing that this matrix is produced by PECs.[15] Double staining for the podocytic transgene Thy-1.1 and the proliferation marker Ki-67 proved that there is no proliferation of podocytes during the development of FSGS lesions. Asano *et al.*[16] confirmed these findings in a recent study. These authors have generated a transgenic mouse with podocytes that express LacZ. Injection of immunotoxin; anti-TAC (FU)-PE38, an immunotoxin against the podocytic CD25 antigen, into these Nphs1-Cre/ROSA26-loxP/NEP25 mice, causes podocyte injury characterized by vacuolar degeneration eventually leading to glomerular sclerosis. In this model proliferating epithelial cells accumulated in Bowman's space, as seen in cFSGS. These authors found no evidence for proliferation or transdifferentiation of podocyte-derived cells but did find proliferating PECs migrating onto the visceral site.

A detailed study of FSGS lesions in the kidney of a patient with recurrent idiopathic FSGS[17] revealed that also in humans the proliferating cells in collapsing lesions are PECs.

Although these studies firmly established a role for PECs in the cellular lesion of cFSGS in mice as well as in a patient with idiopathic FSGS, we questioned if similar events would occur in HIV-associated cFSGS. HIV-associated cFSGS is the prototypical example of cFSGS, characterized by a very high proliferation rate. Proliferating cells are by some authors considered to be dysregulated podocytes, but data from the literature are conflicting. Evidence from *in vitro* studies showed that incorporation of HIV-1 Nef induces proliferation of podocytes.[19] However, in mice podocyte-specific expression of HIV-1 Nef does cause podocytes to enter the cell cycle, but does not lead to completion of mitosis.[20] A recent study in a mouse model of HIV-nephropathy (the HIV13FBV mouse) has provided additional support for our conclusions.[21] These mice express HIV-1 genes in podocytes and avid cell proliferation is observed within Bowman's space. The extracapillary proliferating cells adjacent to the PECs lining Bowman's capsule were often positive for Ki-67 and cytokeratin, whereas the cells adjacent to the glomerular basement membrane were rarely positive for Ki-67. No cells were found doubly positive for synaptopodin and Ki-67. From their observations the authors conclude that most proliferating cells in Bowman's space are derived from PECs.[21]

Barisoni *et al.*[7] and Bariety *et al.*[9] proposed that the loss of specific podocyte markers defines a novel dysregulated and proliferating podocyte phenotype and suggests a common pathogenetic mechanism in collapsing FSGS. In our studies, we found that in collapsing idiopathic FSGS and HIV-associated nephropathy, there was disappearance of podocyte markers, consistent with dedifferentiation. Synaptopodin disappeared very early but remarkably CD10/CALLA[22] remained positive longer, to disappear in advanced lesions. Other investigators also observed a variable degree of expression reduction for different podocyte markers, especially in unaffected portions of the tuft in segmentally injured glomeruli, or in glomeruli without any visible collapse or other histologic abnormalities.[7] Our findings are therefore consistent with dysregulation of podocytes but do not support proliferation of podocytes.

To summarize our date, in HIV and pamidronate-associated cFSGS, we do find dedifferentiation of podocytes but most of the epithelial cells in Bowman's space are derived from the parietal epithelium. These findings are similar to what we previously observed in a patient with recurrent idiopathic FSGS in both the segmental collapsing lesions and noncollapsing FSGS lesions with prominent epithelial hyperplasia. Therefore, it can be argued that in all forms of FSGS with prominent epithelial hyperplasia, there is proliferation of PECs and that in this respect collapsing FSGS can be included in the spectrum of FSGS and need not be regarded as a distinct entity caused by a dysregulated podocyte phenotype. It can be hypothesized that a common pathogenetic mechanism applies to all variants of FSGS, and that the type of lesion that is seen depends on the extent of podocyte activation. In FSGS, secondary to hyperfiltration (often perihilar variant), podocyte activation may be focal and mere loss of podocytes with subsequent adherence of PECs to the glomerular tuft results in sclerosis.[5] If podocyte activation is more widespread and acute, as we belief is the case in primary (idiopathic) FSGS, PEC proliferation is more prominent. Presumably, PEC proliferation is the direct result of interactions between activated podocytes and PECs and growth factors produced by podocytes may play an important role. In HIV-associated collapsing FSGS prominent epithelial proliferation follows massive podocyte activation upon integration of the viral genome. In pamidronate collapsing FSGS rapid widespread toxic loss of podocytes may lead to the collapsing phenotype with less PEC proliferation as compared to HIV-associated collapsing FSGS.

In conclusion, most of the proliferating cells in collapsing glomerulopathy are derived from the parietal epithelium. This conclusion holds for all forms of cFSGS, that is, idiopathic, Pamidronate, and HIV-associated FSGS.

## MATERIALS AND METHODS

### Patients

We studied biopsies from two HIV patients with collapsing FSGS and a nephrectomy specimen from a patient with pamidronate-associated collapsing FSGS. The latter patient was treated with aminohydroxypropylidene bisphosphonate (APD:'pamidronate') because of persistent hypercalcaemia after renal transplantation, she received oral APD in dayly dosage of 300 mg for 20 months, 450 mg for 6 months and 600 mg for 2 months. Subsequently, i.v. APD was administered in a dose of 60 mg at two-weekly intervals. Severe proteinuria was noted after the third infusion. The HIV biopsies contained 11 and 17 different glomeruli, respectively, and the paraffin block from the nephrectomy specimen contained over 77 glomeruli. The material was serially sectioned and from each specimen more than 100 sections were examined. One of every five sections was periodic acid Schiff stain stained for LM evaluation and intervening sections were retained for immunostainings.

### LM

For LM, kidney fragments were fixed in formaldehyde, dehydrated, and embedded in paraplast (Amstelstad, Amsterdam The Netherlands). Two micrometer sections were stained with periodic acid Schiff, and with silver methenamine.[23]

### Immunohistochemistry

Immunohistochemical staining was performed on kidney sections fixed in 4% buffered formaldehyde for 24 h and embedded in paraffin. Four micrometer sections were incubated with monoclonal antibodies (mAbs) and polyclonal antibodies directed at various markers for podocytes, PECs, as detailed in Table 1. As secondary antibody we used power vision Poly-HRP–anti-mouse/rabbit/rat immunoglobulin (IgG) (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of peroxidase as label and diaminobenzidine as substrate.

For the doublestaining CD10/CK8, first the polyclonal antibody CD10 was detected and completed. As detecting antibody we used Envision AF-anti Rabbit IgG (Immunologic, Klinipath, Duiven, The Netherlands), detection was carried out with the use of alkaline phosphatase as label and FastBlue as substrate. Thereafter samples were incubated with the mAb CK8, as detecting antibody we used powervision Poly-HRP-anti Mouse/Rabbit/Rat IgG (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of peroxidase as label and 3-amino-9-ethyl-carbazole/red as substrate.

For the doublestaining Synaptopodin/CK8, first the mAb Synaptopodin was incubated and as detecting antibody we used powervision Poly-HRP-anti Mouse/Rabbit/Rat IgG (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of peroxidase as label and diaminobenzidine as substrate. Thereafter samples were incubated with the mAb CK8, as detecting antibody we used powervision Poly-AP-anti-Mouse IgG (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of alkaline phosphatase as label and 3-amino-9-ethyl-carbazole/red as substrate.

### Immunofluorescence microscopy

Kidney fragments were snap-frozen in liquid nitrogen, and 2 µm acetone fixed cryostat sections were used. Kidney sections were incubated with antibodies directed against heparan sulfate species (Table 1). The single-chain antibodies used for staining of heparan sulfate species[24,25] were detected via a rabbit antibody directed against the VSV-g epitope tag (ICL, Oregon, USA) and finally detected with a goat anti-rabbit Alexa™ 488 antibody (Molecular probes Inc, Leiden, The Netherlands). The sections were examined with a fluorescence microscope (Leica microsystems GmbH, Heidelberg, Germany).

### *In situ* hybridization

Kidney sections were fixed in 4% buffered formaldehyde for 24 h and embedded in paraffin. Four micrometer sections were incubated and subjected to VEGF *in situ* hybridization using a digoxigenin-labeled VEGF-A antisense RNA probe,[26] as secondary antibody we used sheep anti-digoxigenin alkaline phosphatase. Detection was carried out with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-nido-lyphosphate p-tolsaff, the corresponding sense probe was used as a control. This *in situ* hybridization was followed by immunohistochemical staining with the mAb CK8. As secondary antibody we used biotin-labeled rabbit anti-mouse (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of ABC Vectastain, peroxidase as label and 3-amino-9-ethyl-carbazole/red as substrate.

### Transmission electron microscopy

For electron microscopy, we used immersion fixation, small fragments of cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C and washed in the same buffer. The tissue fragments were postfixed in palade-buffered 2% OsO4 for 1 h, dehydrated, and embedded in Epon812, Luft's procedure (Merck, Darmstadt, Germany). Ultrathin sections were contrasted with 4% uranyl acetate for 45 min and subsequently with lead citrate for 5 min at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

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## SUPPLEMENTARY MATERIAL

**Figure S1.** *In situ* hybridisation of mRNA VEGF in (**a**, **b**) normal human and (**c**) mice glomeruli.

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