

## Cellular phenotypes in human stenotic lesions from haemodialysis vascular access

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### Abstract

**Background.** Haemodialysis vascular access dysfunction (due to venous stenosis and thrombosis) is a leading cause of hospitalization and morbidity. The aim of the current study was to identify the specific cell types present within stenotic tissue samples from patients with AV fistula and graft failure.

**Methods.** Discarded tissue segments were collected from the stenotic portions (usually near the graft-vein anastomosis or the AV anastomosis) of 23 dialysis grafts and 20 AV fistulae, and examined for expression of smooth muscle alpha actin, desmin, vimentin and a macrophage marker.

**Results.** The majority of cells within the venous neointima (both grafts and fistulae) were myofibroblasts, with a smaller number of desmin positive smooth muscle cells. The graft neointima had a similar cellular phenotype, albeit without any desmin positive contractile smooth muscle cells. The majority of cells within the PTFE graft material were macrophages. Analysis of sequential sections revealed the presence of fibroblasts within the venous neointima and intragraft region.

**Conclusions.** Our results demonstrate that contractile smooth muscle cells, myofibroblasts, fibroblasts and macrophages all play a role in the pathogenesis of dialysis access dysfunction (grafts and fistulae). Targeting these specific cell types might result in the development of novel therapeutic paradigms for haemodialysis vascular access dysfunction.

**Keywords:** cellular phenotypes; dialysis access; venous neointimal hyperplasia

### Introduction

Haemodialysis vascular access dysfunction is currently a major clinical problem for the more than 300 000 patients on haemodialysis in the United States. It is an important cause of hospitalization and morbidity in haemodialysis

patients, and its economic cost is estimated at well over a billion dollars per annum [1–4]. The vast majority of haemodialysis vascular access dysfunction is due to (a) venous stenosis and thrombosis at the graft-vein anastomosis of PTFE dialysis grafts, as a result of venous neointimal hyperplasia (VNH) (b) venous segment stenosis in AV fistulae that results in both early (failure to mature) and late failures. We and others have previously shown that a key feature of VNH in dialysis access grafts and fistulae is smooth muscle cell proliferation and migration [5,6]. In addition, the later venous stenosis in AV fistulae has previously been shown to be as a result of VNH [7,8], and more recently we have shown that this might also play a role in early fistula failure [9]. Over the last few years, there has been a lot of interest in the concept of the ‘active adventitia’. Thus, a number of authors, including Shi *et al.* [10–12], have performed elegant studies in which they demonstrate the translocation of adventitial fibroblasts (vimentin positive, actin negative cells) through the media and into the intima in experimental coronary angioplasty and saphenous vein bypass graft models [13–19]. Once in the intima, these cells transform into myofibroblasts (vimentin and actin positive) and contribute to neointimal hyperplasia. More recently, it has been suggested that many of the actin positive cells within the neointima in a variety of experimental models of neointimal hyperplasia are in fact bone-marrow-derived stem cells that have acquired a smooth muscle cell phenotype [20]. In the specific setting of dialysis access stenosis, there is currently no information about whether (i) the smooth muscle alpha actin positive cells that we and others have demonstrated within the neointima in tissue specimens from patients with dialysis access stenosis are myofibroblasts or ‘classical’ medial smooth muscle cells with a contractile phenotype, (ii) these cells derive from the adjacent media [21,22], the adventitia [10] or the bone marrow [20]. In view of the current lack of effective therapies for dialysis access stenosis, we believe that these are important questions that could influence the development of novel targeted cell therapies for haemodialysis vascular access dysfunction.

**Table 1.** Schema for cellular phenotyping.

	SMA	Vimentin	Desmin	PGM-1
SMCs	+	–	+	–
Myofibroblasts	+	+	–	–
Fibroblasts	–	+	–	–
Macrophages	–	+	–	+

PGM-1 = macrophage marker.

The aim of this study, therefore, was to characterize the specific cell populations (contractile smooth muscle cell, myofibroblast, fibroblast or macrophage) within the neointima and actual PTFE graft material, in tissue samples from patients with stenotic PTFE grafts or arteriovenous fistulae.

## Materials and methods

### Collection of samples

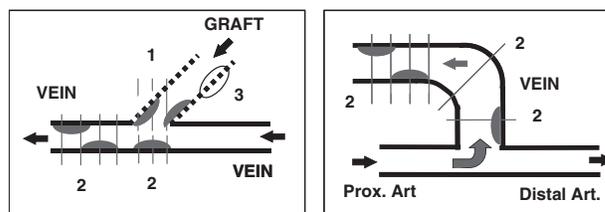
A discarded tissue from the venous segments of 20 PTFE grafts and 23 AV fistulae was collected at the time of access revision surgery. Our surgeons perform both endovascular and open surgical procedures in the operating room, with surgical revision being performed if the endovascular approach fails or is not thought to be clinically appropriate. While previous percutaneous angioplasty may have been performed in some patients, tissue specimens could of course only be obtained at the time of surgery. Although we tried to obtain upstream graft (just before the graft-vein anastomosis) and downstream (proximal) vein (beyond the graft-vein or arteriovenous anastomosis and towards the heart) in each of our samples, this was not always possible due to the surgical constraints associated with refashioning the dysfunctional arteriovenous access (graft or fistula). Demographic and clinical vascular access information was collected whenever possible. Unfortunately, this data were not available for all the specimens. Excised tissue was fixed in formalin and then embedded in paraffin using standard techniques. The number of tissue blocks from each sample varied from 1 to 6. This was dependent on the amount of stenotic venous tissue (for AV fistulae) or graft with attached vein (for PTFE grafts) that was available at the time of access revision surgery. Each block was 3–4 mm in thickness.

### Histological and immunohistochemical analyses

Sections from each tissue block were assessed with a haematoxylin and eosin stain. In addition, sections were examined (with a standard streptavidin biotin immunohistochemical technique) for the expression of alpha smooth muscle actin (DAKO; 1A4; 1:200), desmin (1:400), vimentin (Vimentin, DAKO, 1:1000) and a macrophage marker (DAKO PGM-1, 1:50). Briefly, following deparaffinization and hydration, slides were washed and underwent protease digestion (if required for a particular primary antibody). The slides were then incubated with the primary antibody for 1 h, with the biotinylated secondary antibody blend (anti rabbit Ig, anti mouse IgG and anti mouse IgM) for 30 min and with streptavidin/horseradish peroxidase for 30 min. All incubations were performed at room temperature with appropriate washes between each step. The slides were then developed with a diaminobenzidine/hydrogen peroxide mixture for 4 min, counterstained with haematoxylin, dehydrated with graded alcohols and xylene, and mounted using a xylene based medium. A brown colour indicated a positive stain. Negative controls were performed on each run, by substituting the primary antibody with a relevant non-specific immunoglobulin (rabbit polyclonal or mouse monoclonal) or PBS. In addition, positive control tissue (gut, lymph node and spleen) was used to document the efficacy of each antibody.

### Identification of cellular phenotypes

Table 1 summarizes how the combination of these stains on sequential sections can be used to differentiate between contractile medial-like smooth



**Fig. 1.** Anatomical location of graft and venous neointima: For the purposes of this study, graft neointima is defined as neointima in a direct physical contact with the PTFE graft material (Region 1 in a). Venous neointima is defined as true venous neointimal hyperplasia within the venous segment that is downstream of either the graft-vein anastomosis in PTFE grafts (Region 2 in a) or the arteriovenous anastomosis in AV fistulae (Region 2 in b).

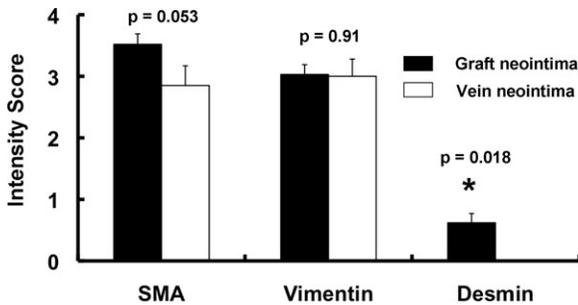
muscle cells (desmin +ve, SMA +ve, vimentin –ve, macrophage –ve), myofibroblasts (desmin –ve, SMA +ve, vimentin +ve, macrophage –ve), fibroblasts (desmin –ve, SMA –ve, vimentin +ve, macrophage –ve) and macrophages (desmin –ve, SMA –ve, vimentin +ve, macrophage +ve). While there remains some controversy about the best combination of markers for the cellular phenotyping of vascular cells, a number of published papers support the use of such a schema [10,13,23].

### Semi-quantitative scoring system

Sections from the patients with AV fistula stenosis (Figure 1b; Region 2) were scored for the percentage of positive neointimal cells for each of the specific markers described above as a percentage of the total number of neointimal cells. For tissue specimens that had multiple blocks, an average assessment (subjective) for all the tissue blocks was made. Tissue blocks from the PTFE graft patients were first assessed to see if there were sections with PTFE graft. These PTFE graft sections were assessed separately for (a) the percentage of positive neointimal cells for the specific markers described above as a percentage of the total number of neointimal cells (Figure 1a; Region 1) and (b) the percentage of positive intragraft cells for the specific markers described above as a percentage of the total number of intragraft cells (Figure 1a; Region 3). Tissue blocks from patients with PTFE grafts with venous tissue sections only (Figure 1a; Region 2, downstream or proximal from the graft) were then assessed as for the AV fistula specimens. All tissue specimens were examined by a pathologist (LA). A semi-quantitative scoring scale from 0 to 4± was used to quantify the percentage of positive cells for a specific marker as compared to the total number of cells (0–10% = 0; 11–25% = 1±; 26–50% = 2±; 51–75% = 3± and 76–100% = 4±). Individual cells were not counted since the percentage of positive cells was often very different within different portions of the same specimen. Selection of some fields only for counting could therefore have introduced bias into our results. As described above, this analysis was performed for venous neointima (Region 2 in Figures 1a and b), graft neointima (Region 1 in Figure 1a) and for intragraft cells (Region 3 in Figure 1a). We chose to classify regions in this way, since this is a study that is based around histological specimens rather than angiographic data. We were also very interested in attempting to identify the impact of the presence of the PTFE graft material on neointimal hyperplasia, through the comparison of cellular phenotypes within the neointima overlying the graft (Figure 1a; Region 1) and that within downstream vein (Figure 1a; Region 2).

## Results

Cellular phenotypes were assessed in three different regions as described in Figure 1. In the case of PTFE grafts, these regions were (a) graft neointima (Region 1 in Figure 1a) (b) venous neointima (Region 2 in Figure 1a) and (c) intra-graft cells (Region 3 in Figure 1a). In the clinical setting



**Fig. 2.** A comparison of graft and venous neointima in AV fistulae and PTFE grafts: both graft and venous neointima (from the venous segment of patients with PTFE grafts and AV fistulae) appear to comprise SMA +ve, vimentin +ve myofibroblasts albeit with a small but a well-documented (and statistically significant) presence of contractile smooth muscle cells in the venous (but not graft) neointima. Please note that the venous neointima in this graph includes the vein that is downstream to the AV anastomosis or graft-vein anastomosis in both AV fistulae and AV grafts.

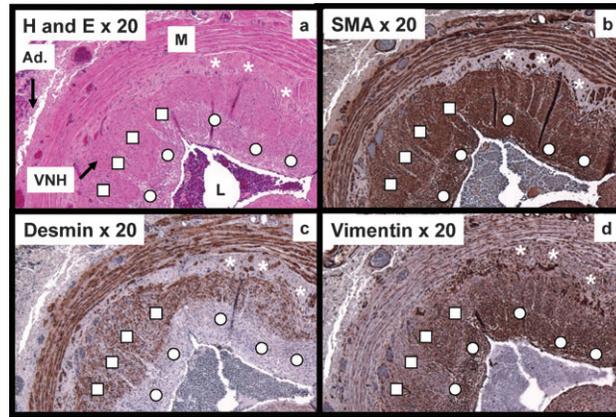
of AV fistulae, we assessed cellular phenotypes for venous neointima only (Region 2 in Figure 1b).

#### Clinical information

Clinical information about the type of access was available for 28 tissue specimens (9 grafts and 19 AV fistulae). Of the 19 AV fistulae, 8 were radiocephalic, 6 were brachiocephalic and 5 were brachio basilic transpositions. The time interval between creation of the access and surgical removal in these studies showed wide variations (26–1042 days for radiocephalic AV fistulae; 113–1170 days for brachiocephalic AV fistulae; 146–384 days for brachio basilic transpositions and 41–704 days for PTFE grafts). Thus, the AV fistula specimens studied in these experiments represented both early and late failures.

#### VNH in the stenotic downstream venous segment of AV Fistulae and PTFE Grafts comprises primarily myofibroblasts with some contractile desmin positive smooth muscle cells

Figure 2 describes the semi-quantitative cellular phenotyping scores for all the specimens with venous neointima hyperplasia without the presence of PTFE graft within that particular section (this would comprise downstream vein beyond the graft-vein anastomosis (Region 2 in Figure 1a) or arteriovenous anastomosis (Region 2 in Figure 1b) of AV grafts and fistulae, respectively). These results are described by the black legend in Figure 2. Note that the vast majority of cells within the venous neointima of both dysfunctional AV fistulae and grafts are SMA +ve (score of  $3.53 \pm 0.18$ ), vimentin +ve (score of  $3.03 \pm 0.16$ ) myofibroblasts. There are also a small number (score of  $0.63 \pm 0.15$ ) of desmin +ve contractile smooth muscle cells within the venous segment of stenotic AV fistulae or grafts. Figure 3 describes the actual histology from a representative specimen with VNH, documenting the presence of fibroblasts, myofibroblasts and contractile smooth muscle cells within the venous neointima.



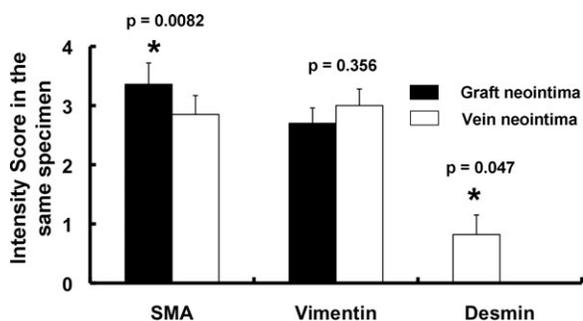
**Fig. 3.** Venous neointimal hyperplasia in dialysis access dysfunction: this figure describes representative sequential sections from a failed arteriovenous fistula stained with H and E (a), SMA (b), desmin (c) and vimentin (d). Using the classification from Table 1, the white asterisks denote a region of fibroblasts (SMA –ve, desmin –ve, vimentin +ve cells), the white circles a region of myofibroblasts (SMA +ve, desmin –ve, vimentin +ve cells) and the white squares a region of contractile smooth muscle cells and myofibroblasts (SMA +ve, desmin +ve, vimentin –ve contractile smooth muscle cells, with also presumably some SMA +ve, desmin –ve, vimentin +ve myofibroblasts). Ad. = adventitia, M = media, VNH = venous neointimal hyperplasia.

#### Neointima overlying PTFE Graft comprises primarily myofibroblasts without any contribution of contractile desmin positive smooth muscle cells

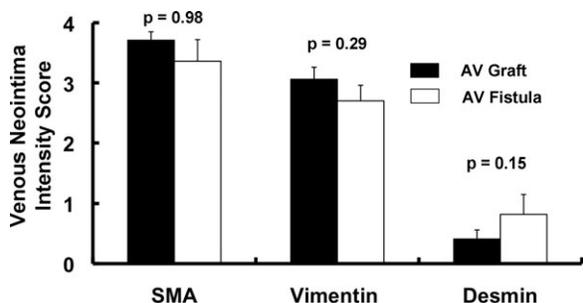
Figure 2 also describes the comparative scores for the graft neointima (red legend in Figure 2) that overlies the PTFE graft material (Region 1 in Figure 1a). Note that these cells are also predominantly SMA +ve and vimentin +ve myofibroblasts. In contrast to the cellular phenotype of venous neointima, graft neointima has no desmin staining (desmin score =  $0.00 \pm 0.00$ ), suggesting the complete absence of contractile smooth muscle cells in this setting.

#### Comparison of the cellular phenotype of graft and venous neointima in the same PTFE graft

In order to remove the confounding effects of the presence or absence of the PTFE graft material in determining the composition of the cellular phenotype of venous neointima, we also compared the cellular phenotype of graft neointimal (Region 2 in Figure 1a) and venous neointimal (Region 1 in Figure 1a) hyperplasia in specimens from the same AV graft. Neointimal hyperplasia that was in physical contact with PTFE graft was defined as graft neointimal (Figure 1a; Region 1) while neointimal hyperplasia that was not in direct contact with PTFE graft (albeit from the same patient) was defined as venous neointima in patients with a PTFE graft (Figure 1a; Region 2). All these tissue samples were removed surgically although patients may have had previous angioplasties. Figure 4 documents that venous neointima downstream from PTFE graft (Region 1 in Figure 1a) tends to have significantly greater scores for SMA and desmin as compared to graft neointima (Region 2 in Figure 1a) from the same patient, once again suggesting the presence of contractile smooth muscle cells as well as myofibroblasts



**Fig. 4.** A comparison of graft and venous neointima in PTFE grafts only: as for the larger groups of venous neointima versus graft neointima in Figure 2, the main finding from this analysis of PTFE grafts alone was the presence of desmin+ve cells within the stenotic venous segment downstream of PTFE grafts but NOT within graft neointima (in physical contact with PTFE graft on the histological sections).



**Fig. 5.** A comparison of venous neointima in AV fistulae and AV grafts: this figure demonstrates no difference in the cellular phenotype of venous neointima in AV fistulae (venous segment downstream of the AV anastomosis) or AV grafts (venous segment downstream of the graft-vein anastomosis).

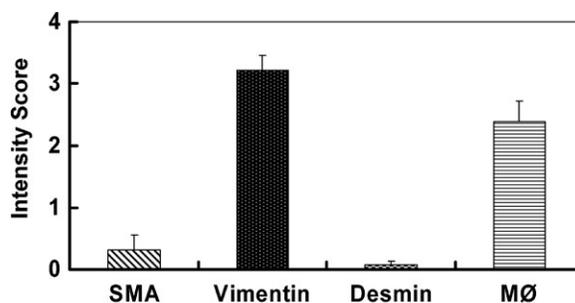
in venous neointima downstream to the graft-vein anastomosis, but not within graft neointima (predominantly myofibroblasts).

#### *The cellular phenotype of venous segment stenosis in AV grafts and AV fistulae is similar (myofibroblasts with a few contractile smooth muscle cells)*

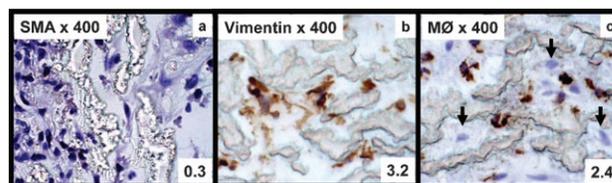
Figure 5 compares the cellular phenotype of venous segment stenosis (Region 2 in Figure 1a and b), in AV fistulae and AV grafts. Note that there are no differences ( $P > 0.15$ ) in the cellular phenotype (predominantly SMA +ve, vimentin +ve myofibroblasts) in both these settings.

#### *Intragraft cells*

Figure 6 describes the phenotype of intragraft cells. Note that the vast majority of intragraft cells are positive for vimentin and a macrophage marker suggesting that these cells are primarily macrophages. There is an almost complete absence of desmin and SMA positive cells within the graft material.



**Fig. 6.** Cellular phenotyping of intragraft cells: this figure documents that the vast majority (but not all; see Figure 7) of the cells within the PTFE graft material are macrophages (these cells also express vimentin).



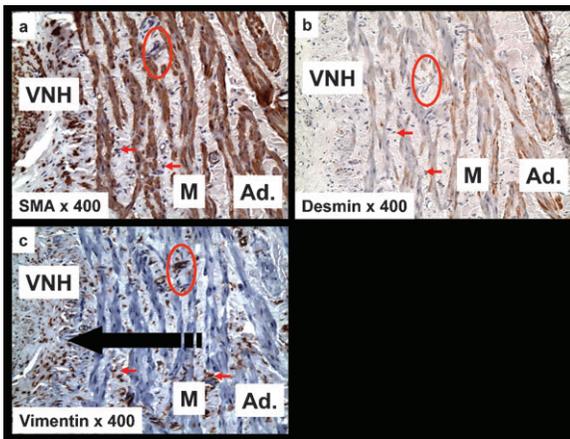
**Fig. 7.** Cellular phenotype of intragraft cells: this figure shows sequential sections of PTFE graft stained for SMA (a), vimentin (b) and a macrophage marker (c). Note that all the cells are positive for vimentin and negative for SMA, but that only some of these cells are macrophages (c). All these cells were negative for desmin (data not shown). It is likely that the vimentin +ve and macrophage -ve cells are intragraft fibroblasts (arrows in c). Inset figures denote the semi-quantitative scores from Figure 6.

#### *Fibroblast-like cells within PTFE graft, venous media and venous neointima*

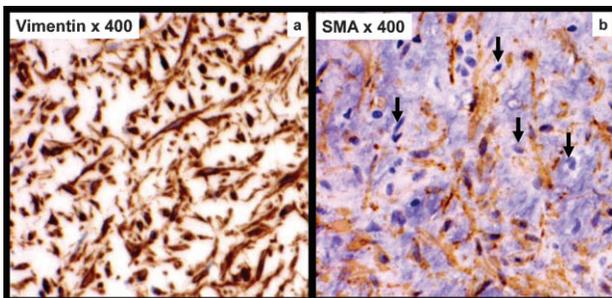
Although the data presented in the semi-quantitative scoring analysis in Figures 2, 4 and 5 do not suggest the presence of fibroblasts, examination of some sequential sections of PTFE graft (Figure 7), venous media (Figure 8) and venous neointima (Figure 9), clearly document the presence of vimentin +ve, desmin -ve, SMA -ve cells that are likely to be fibroblasts in all these settings. A similar pattern also emerges from Figure 3, where the white asterisks represent a region of SMA -ve, desmin -ve, vimentin +ve fibroblasts.

## **Discussion**

We have described for the first time, a detailed analysis of the actual cellular phenotype that is present within the venous stenosis that characterizes haemodialysis vascular access dysfunction. Our results suggest that VNH (venous neointima) in the setting of dialysis access grafts and fistulae is primarily composed of vimentin +ve, SMA +ve, desmin -ve myofibroblasts rather than desmin +ve contractile smooth muscle cells. This is in keeping with a similar presence of myofibroblasts in experimental venous bypass graft models [24]. Our studies have also been able to demonstrate that venous neointima (both in the setting of AV fistulae and PTFE grafts) does have a small proportion of desmin +ve cells that indicates a contractile smooth muscle phenotype. This was not the case, however, for graft neointima, which was in a direct physical contact



**Fig. 8.** Cellular phenotype of media infiltrating cells: this figure describes sequential sections of the medial layer from a patient with AV fistula stenosis, stained for the presence of SMA (a), desmin (b) and vimentin (c). Note the presence of SMA –ve, desmin –ve, vimentin +ve cells (? fibroblasts) within the circle and identified by arrows within the smooth muscle cell bundles. We believe that the presence of such cells with a fibroblast-like phenotype suggests (indirectly) that adventitial fibroblasts could be migrating from the adventitia (through the media) to the intima (c). These cells were not seen in such numbers on examination of normal vein. VNH = venous neointimal hyperplasia, M = media, Ad. = adventitia.



**Fig. 9.** Cellular phenotype of neointimal cells: this figure shows a sequential high power views of venous neointima stained for vimentin (a) and SMA (b). All these cells were desmin –ve. Note the presence of vimentin +ve, SMA –ve, desmin –ve cells that are likely to be fibroblasts (arrows in Figure 9).

with the PTFE graft material. In addition, we were able to demonstrate (through the use of sequential section stains) the presence of desmin –ve, SMA –ve, vimentin +ve cells (possibly fibroblasts) within the PTFE graft material, the venous media and the venous neointima, in at least some of our specimens.

What do these results tell us about the pathogenesis of VNH in the specific setting of dialysis access dysfunction and how could these findings lead to the development of novel therapeutic interventions for this clinical problem?

#### *Adventitial migration*

The presence of fibroblast-like cells within the interstices of the graft and also within the venous media suggests that there could be a migration of fibroblasts from the adventitia, through the media and/or PTFE graft, into the neointima as has been previously described in experimental models of

coronary angioplasty and vein bypass grafting [10,11,13, 17]. In addition, a recent study using BrdU staining in a pig arteriovenous graft stenosis model also suggested a migration of cells from the adventitia into the intima [25]. In support of these pathogenetic studies, Meng *et al.* [26] have recently demonstrated that the adventitial application of gene therapy for a tissue inhibitor of metalloproteinase (TIMP) can reduce neointimal hyperplasia, while our own group has demonstrated a significant reduction in luminal stenosis in a pig arteriovenous graft stenosis model with the use of perivascular paclitaxel eluting wraps [27]. Thus, the clinical relevance of our current findings is that, in combination with the experimental studies described above, it provides a biological rationale for the development of perivascular-based therapies for dialysis access dysfunction. Studies on the use of perivascular therapies in the setting of dialysis access grafts and fistulae are currently in Phase II/III trials in the United States. These results also suggest that developing novel forms of a graft material that prevent the migration of adventitial cells may also be effective in reducing the volume of graft and venous neointima in dialysis access grafts and fistulae. These approaches would not be as effective, however, against bone-marrow-derived progenitor cells that bind to the site of vascular injury and then differentiate into smooth muscle cells and myofibroblasts. The exact role for these bone marrow derived progenitor cells in the pathogenesis of dialysis access stenosis is unclear, although a recent paper by Caplice *et al.* documents a role for bone-marrow-derived cells in a rat model of AV fistula stenosis [28].

#### *Differentiation and dedifferentiation within the venous and graft neointima*

By demonstrating that there is a mix of different cell types within venous (contractile smooth muscle cells, myofibroblasts and fibroblasts) and graft (myofibroblasts and fibroblasts) neointima, our research opens up the possibility that there could be ongoing phenotype switching within the lesion of VNH in dialysis access grafts and fistulae. Thus, it is possible that the migrating fibroblasts described above could acquire SMA expression to become myofibroblasts, or alternatively, contractile smooth muscle cells migrating from the media could lose desmin expression and acquire vimentin expression, thus transforming into myofibroblasts. In addition, it is possible that such phenotype switching may be an ongoing process, even within what would be considered to be mature venous or graft neointima. At present, there is very limited information about these processes in the specific setting of dialysis access grafts and fistulae. In particular, there is no information about the temporal sequence for the presence or absence of these different cellular phenotypes following the placement of a new PTFE graft or AV fistula.

In conclusion, we have described for the first time a detailed evaluation of the different cellular phenotypes involved in the pathogenesis of dialysis graft and fistula stenosis. Our results (a) emphasize the role of the myofibroblast as the predominant cell type involved in the pathogenesis of VNH (similar to the data described by us in the context of

early AV fistula failure [9]), (b) draw attention to a possible migration of adventitial fibroblasts from the adventitia to the intima through the venous media or through PTFE graft, (c) underscore our current lack of understanding about possible phenotype switching within the venous neointima and (d) suggest that targeting of specific cell types could be possible future novel therapies for haemodialysis vascular access dysfunction.

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**Conflict of interest statement.** None declared.

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