

Cell distribution and regenerative activity following meniscus replacement

Cathal J. Moran · Selma Atmaca · Heidi A. Declercq ·
Maria J. Cornelissen · Peter C. Verdonk

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Abstract

Purpose Meniscus replacement is of clinical benefit, but universal efficacy remains elusive. A greater understanding of the biological activity within implanted allografts or synthetic scaffolds may assist the development of improved surgical strategies.

Materials Biopsies of fresh–frozen allograft ($n=20$), viable allograft ($n=18$) and polyurethane scaffolds ($n=20$) were obtained at second-look arthroscopy. Histological evaluation of tissue morphology and cell density/distribution was performed using haematoxylin–eosin (H&E) staining. Immunohistochemistry was used to detect the presence of CD34 (on progenitor cells and blood vessels) and smooth muscle actin (SMA)-positive structures and aggrecan. Collagen presence was investigated using picrosirius red staining.

Results Cell density in the deep zone of the meniscus replacement was significantly higher in polyurethane scaffolds versus allograft transplants ($p<0.01$) and also significantly higher in viable allograft compared with deep-frozen allograft ($p<0.01$). CD34 staining was significantly higher in polyurethane and viable allografts versus deep-frozen allograft (progenitor cells $p<0.05$; blood vessels $p<0.01$). There were no significant differences in SMA or aggrecan staining across groups. All three specimen types demonstrated strong presence of collagen type I.

Conclusions Both viable allograft and a polyurethane meniscal scaffold show enhanced morphological, cell-distribution and regenerative patterns over deep-frozen allograft following

surgical implantation. Given the limitations in viable allograft availability, these findings support the continued development of synthetic scaffolds for meniscus replacement surgery.

Keywords Meniscus · Replacement · Allograft · Histology · Regeneration

Introduction

Meniscal pathology accounts for approximately 1.5 million surgeries across the United States and Europe annually [1, 2]. Although meniscectomy often relieves symptoms in the short term, long-term follow-up indicates that meniscal removal provides an increased risk of osteoarthritis [3, 4]. While meniscal repair is growing in popularity, its role is relatively limited given the inherent low vascularity of the inner zones of the meniscus [5, 6]. Furthermore, repair in the outer vascularised zones is known to fail in up to 25 % of cases [7]. Meniscal replacement surgery uses natural or synthetic scaffolds to guide tissue repair or regeneration in three dimensions while providing a temporary construct for mechanical function. Regeneration of meniscal tissue is believed to occur via recruitment of cells from adjacent tissues (meniscal remnant and synovial membrane). Tissue engineering may also have a role through the additive benefit that cells or growth factors may bring [8, 9]. Regardless of the strategy employed, creating a permissive microenvironment for host-cell attachment, proliferation and matrix synthesis is the desired effect of meniscal replacement.

Clinical application of meniscus allograft transplantation has shown clinical efficacy for symptomatic meniscal deficiency at intermediate term, with important long-term survival analysis data now also supporting this [10, 11]. The clinical use of synthetic replacement scaffolds also shows promising early results for symptomatic partial meniscal defects [12–14].

C. J. Moran · P. C. Verdonk (✉)
Antwerp Orthopaedic Centre, Monica Hospitals, Harmoniestraat 68,
Antwerp 2018, Belgium
e-mail: pverdonk@yahoo.com

S. Atmaca · H. A. Declercq · M. J. Cornelissen
Department of Basic Medical Sciences, Ghent University, Ghent,
Belgium

However, meniscus replacement (allograft transplantation or scaffold implantation) does not demonstrate universal clinical efficacy in the short or medium term and has not been proven to have a significant impact on the natural history of the meniscus-deficient knee in terms of degenerative change over time. As the long-term efficacy of allografts and scaffolds is likely to be influenced by implant population with host cells and the onset of regenerative activity, information on this process as it occurs in the clinical environment is important. The native meniscus exhibits significant spatial variations in cell distribution, vascularity and healing activity. However, little is known about the source, nature and distribution of cells that contribute to tissue regeneration following meniscal replacement surgery. CD34+/CD31 cells were noted to be present in the superficial zone of the normal meniscus [15]. CD34 is a recognised progenitor-cell marker, supporting the idea that these meniscal cells may have a role in tissue homeostasis and repair/regeneration following surgery [16]. The presence of contractile cell phenotypes and vascular activity are also believed to be a feature of regenerative activity or potential. Increased expression of contractile anti-smooth muscle actin (a-SMA) isoform in both meniscus cells and vascular smooth muscle is seen in the healing process in injured meniscus [15, 17]. The presence of collagen and proteoglycan (aggrecan; denoting matrix formation) are also indicative of cellular activity and possible meniscal regeneration.

The purpose of this study was to evaluate histological and immunohistochemical features of human clinical meniscal tissue following meniscus allograft transplantation (deep-frozen and viable allograft) and synthetic polyurethane meniscus scaffold implantation.

Materials and methods

Patients

The study group comprised 58 patients who underwent prior meniscus replacement surgery at our institute. All patients had clinical symptoms consistent with meniscal deficiency prior to meniscus replacement surgery. Biopsies of fresh-frozen allograft ($n=20$), viable allograft ($n=18$) and replacement polyurethane scaffolds ($n=20$) were obtained from patients at a mean of 18 months following surgery. For meniscus replacement with allograft (in patients who were symptomatic due to total or subtotal meniscal deficiency) or a polyurethane scaffold (in patients symptomatic due to partial meniscal deficiency; Actifit, Orteq, UK), a similar soft-tissue surgical technique was applied. All patients underwent partial or total meniscectomy with surgical debridement back to the vascularised zone of the damaged portion of the

meniscus. The appropriately sized implant was placed into the knee joint through the anteromedial or anterolateral portal and sutured to the native meniscus. The suturing techniques employed were all-inside, inside-out, or outside-in methods, depending on the area being sutured. To ensure protection of the newly formed fragile tissue and to provide optimum conditions for healing, all patients were required to undergo a conservative rehabilitation programme. The rehabilitation protocol was followed for 16–24 weeks, with the patient non-weight bearing for the first three weeks. Partial weight bearing was permitted from week four onwards, with a gradual increase in loading up to 100 % load at nine weeks after implantation. Full weight bearing with an unloader brace was allowed from week nine onwards and without the use of the unloader brace from week 14 onwards. Gradual resumption of sports was generally commenced as of 6 months.

Tissue biopsy and analysis

For the purposes of histological and immunochemical evaluation, specimens of the implanted meniscal replacement were obtained through 3-mm-diameter biopsies taken during second-look arthroscopy. The center of the inner free edge of the implanted meniscal replacement was chosen for the biopsies because it is the area furthest away from the vascularised native meniscus rim and hence would be the area likely to be populated last by cells. Furthermore, it was concluded that a biopsy in this area would be least likely to damage the meniscal replacement.

Meniscus tissue was fixed in 4 % paraformaldehyde and embedded in paraffin. Five-micrometer paraffin sections were deparaffinised with xylene and then rehydrated in ethanol. H&E staining was performed to visualise meniscus and cell morphology and investigate cell density. Picrosirius red was used for collagen staining. Immunohistochemical staining was performed using monoclonal antibody directed against the cell and extracellular matrix (ECM) markers CD34, SMA and aggrecan (all from DAKO Cytomation, Heverlee, Belgium). Histological and immunohistochemical protocols were used, as previously described [15].

Cell density in the superficial zone (cell layer parallel to meniscus surface) and deep zone was evaluated. The superficial zone was distinguished from the deep zone through the change in tissue structure (change in cell density and/or orientation of ECM). Meniscus biopsies were evaluated for the presence of CD34 and SMA-positive cells and counted per ten fields of 0.65 mm selected at random in the superficial zone parallel to the surface, and the corresponding percentage was calculated. A histological score of 0–3 points was assigned to

the presence of CD34 and SMA-positive cells in each biopsy: (0) 0 %, (1) 0–20 %, (2) 20–40 % and (3) 40–60 % positive cells. CD34-positive vascular structures were also counted per square millimetre (mm²) on anti-CD34-stained sections. There was no distinction made on the basis of diameter of the different vessels. Aggrecan presence was also evaluated: 0=no staining; 1=low intensity, 2=moderate intensity, 3=high intensity. For picrosirius red staining (polarisation microscopy), the scoring system used was as follows: 0=black; 1=predominantly yellow/green (collagen type II); 2=predominantly orange/red (collagen type I); 3=50/50 yellow/red (collagen type II and type I). All analyses were performed blindly by three independent observers.

In addition to the provision of descriptive data for each group, statistical significance between groups was evaluated; the level of significance was set at $p < 0.05$. Data were analysed using SPSS 17.

Results

Cell types visualised on histology of all implants

H&E staining demonstrated the presence of two different cell types in all implant types: fibrochondrocytes (Fig. 1a–c) and fibroblasts (Fig. 1d–f). Fibrochondrocytes have a round morphology with a clear core surrounded with gaps and were most common in the deep zone (Fig. 1a'–c'). Fibroblastic cells have a more stretched morphology (Fig. 1d'–f') and were more common in the superficial zone of the menisci.

Polyurethane meniscus scaffold

H&E staining identified extensive cell colonisation and ECM formation throughout the polyurethane scaffold (Fig. 2). Twelve biopsies had a similar morphology to that shown in Fig. 2a, 3/20 to that in Fig. 2b and 5/20 to that in Fig. 2c, likely linked to variable degradation of the polyurethane scaffold. CD34 cellular immunohistochemistry was positive in 14/20 specimens (Fig. 3a'). Extensive vascularity was demonstrated by SMA-positive staining in the smooth muscle cells around blood vessels, as well as the presence of CD34 staining in vascular structures (Fig. 4a' and b'). ECM in biopsies showed a medium intensity for aggrecan staining (Fig. 4c–c'). Light and polarised microscopy of picrosirius red staining demonstrated a predominantly red colour, illustrating the presence of collagen type I (Fig. 4 d'–e').

Deep-frozen allograft

H&E staining identified two different morphological types in the deep-frozen allograft: eight of 19 had similar morphology

to that shown in Fig. 5a and 11 of 19 similar to that shown in Fig. 5b (Fig. 5a, b). Four specimens showed strong CD34 positivity, one showed weak positivity and 12 showed none. Two biopsies were not scored because slide specimens were lost during CD34 staining. Anti-SMA staining confirmed the presence of vascular structures, but there was limited CD34 vascular staining. A strong intensity for aggrecan staining was shown in this group, as was an intense red colouring on picrosirius red staining, indicating a very high content of collagen type I.

Viable allograft

Biopsies in the viable allograft group could again be classified into two groups on the basis of H&E staining (Fig. 6a, b): eight of 18 were morphologically similar to that shown in Fig. 6a and ten of 18 to that shown in Fig. 6b. CD34 positivity was detected in 12/20. One biopsy could not be interpreted due to a fault with the staining procedure. CD34 and anti-SMA vascular staining also yielded strong positivity. Aggrecan staining was weak, however. Picrosirius red staining identified a predominantly red colour.

Comparison of findings across implant groups

The three populations were compared for statistically significant differences between cell density overall, in the superficial zone and in the deep zone; number of vascular structures identified (including capillaries); and intensity of anti-CD34 and antiaggrecan immunostaining and differences in colour of picrosirius red staining. In terms of overall cell density, mean density was 896 ± 5.77 cells/mm² in the polyurethane scaffold group, 850 ± 6.18 cells/mm² in the viable allograft group and 658 ± 5.31 cells/mm² in the deep-frozen transplant group ($p > 0.05$). When cell density in deep and superficial zones were analysed separately, there was a statistically significant difference in mean cell density in the polyurethane scaffold in the deep zone (896 ± 5.77 cells/mm²) and viable allograft (426 ± 3.71 cells/mm²; $p < 0.01$), and between both of those groups and mean cell density in the deep zone of the frozen allograft group (177 ± 2.1 cells/mm²; $p < 0.01$). There was no significant difference between groups in terms of cell density in the superficial zone. There was, however, a difference in actual cell type present in the superficial zone of the polyurethane scaffold compared with both allograft types. Specifically, the main cell type seen in the polyurethane scaffold was fibrochondrocytes versus fibrochondroblasts in both allograft types.

When CD34 progenitor-cell staining was compared across groups, there was significantly higher staining found for progenitor cells in the viable allografts versus deep-frozen allografts ($p < 0.01$). There was also a higher level of CD34 progenitor-cell staining seen in the polyurethane scaffold

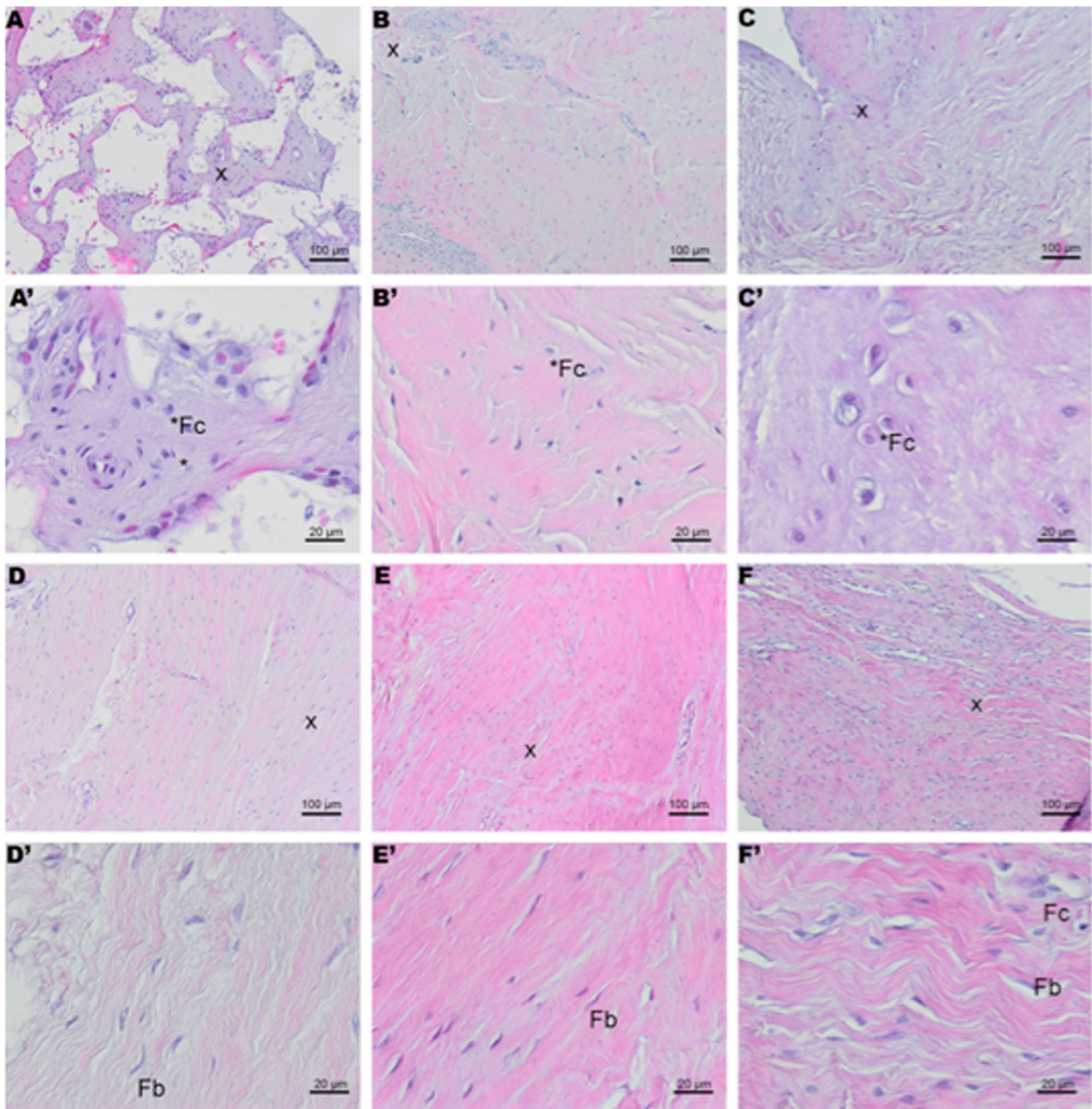


Fig. 1 Haematoxylin and eosin staining of the various populations. *x* zone zoomed in. Biopsies from patients with: **a–a'**, **d–d'** polyurethane scaffold; **b–b'**, **e–e'** deep frozen allograft; **c–c'**, **f–f'** viable allograft. *Fc* fibrochondrocytes; *Fb* fibroblasts

Fig. 2 Three different trends in the morphology found within the polyurethane scaffold implant group. Time of biopsy after implantation: **a** 11 months; **b** 13 months; **c** 26 months

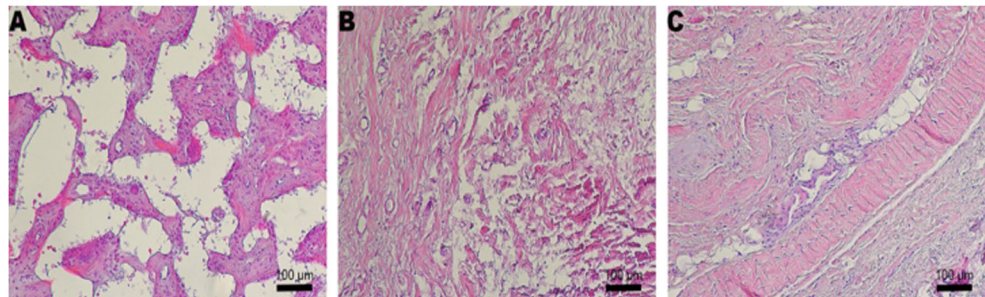
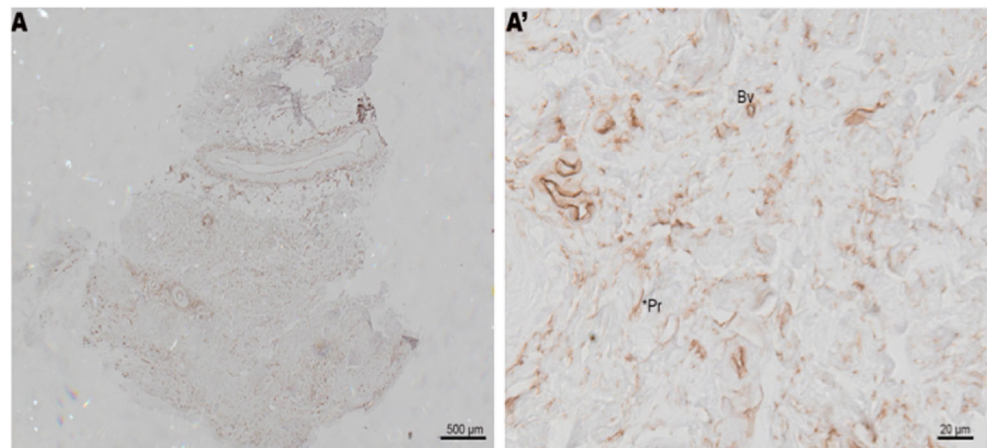


Fig. 3 Biopsy of a patient with a polyurethane implant. The endothelial lining and possible precursor cells are CD34 positive. *Bv* blood vessel; *Pr* progenitor cell



versus deep-frozen allograft, but this did not reach significance ($p=0.06$). CD34 vessel-staining analysis resulted in a statistically significant difference between groups, with both the polyurethane scaffold (158 ± 1.65 vessels/ mm^2) and the viable allograft (241 ± 2.17 vessels/ mm^2) showing a higher number of vessels than the deep-frozen allograft specimens (74 ± 1.50 vessels/ mm^2 ; $p < 0.01$).

There were no extravascular SMA-positive cells identified in specimens in this study. Instead, only the smooth muscle around the vessels was SMA positive, with no significant differences noted across groups. There was also no significant difference in aggrecan staining across groups. Finally, all groups demonstrated the presence of high-intensity staining for collagen type 1, again with no statistical difference noted.

Discussion

Although there is ever-increasing data to support meniscus allograft transplantation and synthetic meniscus scaffold implantation, a successful outcome for these surgical procedures is not universal. Although allograft material may contain some endogenous cells, the implantation of synthetic scaffolds is undertaken on the premise that they can act as a regenerative platform for the cell population from host tissues, with subsequent matrix formation and remodeling prior to degradation of the implanted scaffold. Adequate survival, durability and function of both allografts and synthetic material requires that maximal cell infiltration and subsequent tissue regeneration happens in a timely manner.

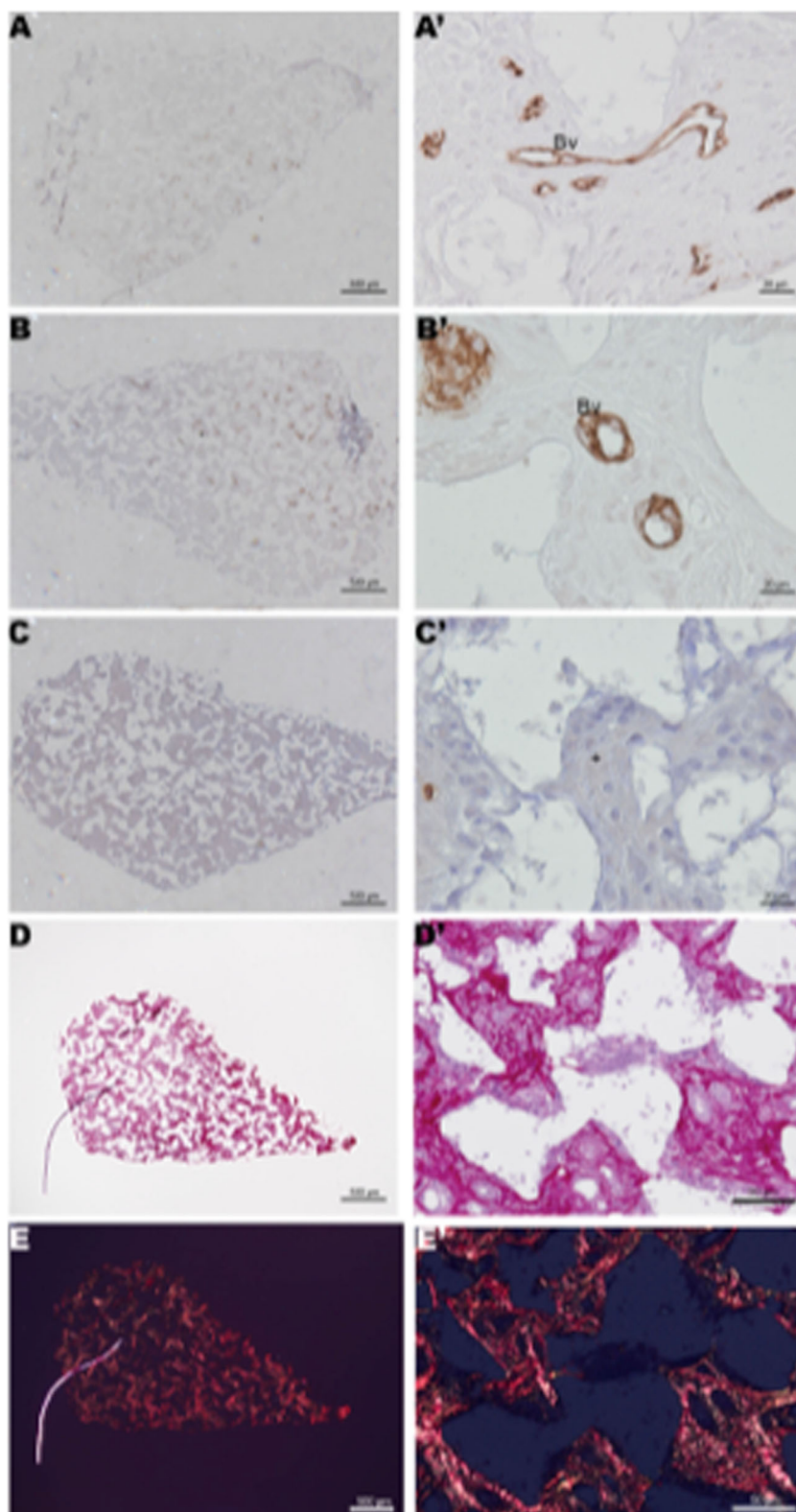
Rodeo et al. published one of the first, and most informing, reports on the phenotype of cells that repopulate meniscal allograft, whether an immune response is elicited against the graft and whether the repopulating cells synthesise normal ECM components [18]. They study found that most specimens in their series, obtained from follow-up arthroscopy, demonstrated incomplete repopulation with viable cells. The

repopulating cells stained positively with phenotype markers for both synovial cells and fibroblasts. Polarised light microscopy demonstrated evidence of active remodeling of the matrix. They also noted that although there is histological evidence of an immune response directed against the transplant, this response does not appear to affect the clinical outcome. Noyes et al. also reported that the cells in biopsy specimens from human meniscal allografts were generally fibroblastic rather than fibrochondrocytic [19]. They examined only biopsy specimens from failed meniscal transplants, however, whereas Rodeo et al. reported on small biopsy specimens from intact transplants [18, 19].

In our study, evidence of both fibroblasts and fibrochondrocytes was found in all specimen types. There was, however, a difference in cell type in the polyurethane scaffold compared with both allograft types. Specifically, the main cell type seen in the polyurethane scaffold was a fibrochondrocyte, most evident in the superficial zone, versus a fibrochondroblast in both allograft types. The significance of this is unclear, but as the native meniscal cell is believed to be fibrochondrocytic in nature, it may point to biological suitability of the polyurethane scaffold for regenerative purposes. However, it has been noted that there is no unique cell-specific marker for meniscal cells. Identifying the exact phenotype of the repopulating cells requires analysis of the RNA produced by these cells in comparison with the RNA produced by normal meniscal fibrochondrocytes [18].

When cell density is considered, density seen in the central area of biopsies from deep-frozen allografts was significantly lower than that seen in the viable allograft and polyurethane scaffold groups. This result is reflected in data from previous reports on deep-frozen allografts, where repopulation was limited to the superficial zone while the core of the transplant remained acellular [1]. This may be related to the processing of the deep-frozen allografts, with loss of cells as a result. Again, this finding suggests a role for ongoing work in the development and application of suitable synthetic scaffolds capable of repopulation following implantation. A recent

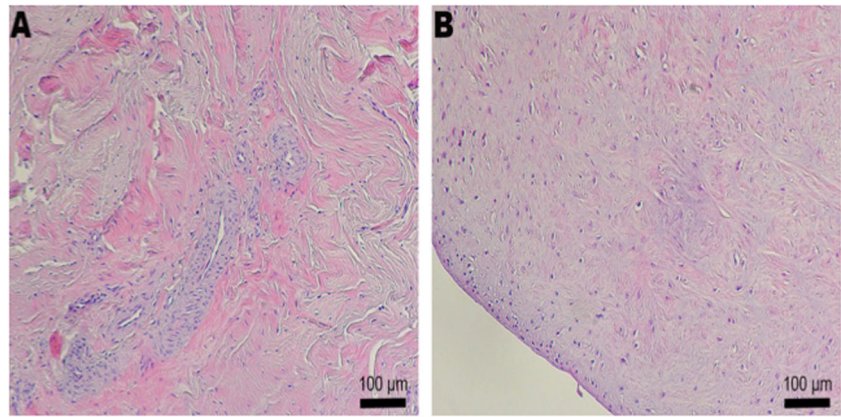
Fig. 4 Immunohistochemistry and picrosirius red staining on one selected patient with a polyurethane scaffold implant. **a, a'** anti-CD34; **b, b'** anti-smooth muscle actin (SMA); **c, c'** anti-aggrecan; **d, d'** picrosirius red staining on light microscopy; **e, e'** picrosirius red staining on polarisation microscopy. *Bv* vessel; Aggrecan staining



report provided further histological data on the polyurethane scaffold from second-look arthroscopy and biopsy in 44 patients at a mean 12-month follow-up [20]. The layered histological structure observed at 12 months was consistent in all 44

biopsy samples, having meniscus tissue-like characteristics specifically in relation to cellular structure and ECM. That report noted the presence of three distinct layers with layer 1, resembling the peripheral red vascularised zone typically rich

Fig. 5 Two different trends in morphology within the population with a deep-frozen allograft transplant. Time of biopsy after implantation: **a** could not be identified; **b** 11 months. Average cell density: **a** 750 ± 3.02 cells/mm²; **b** $1,317 \pm 3.82$ cells/mm²



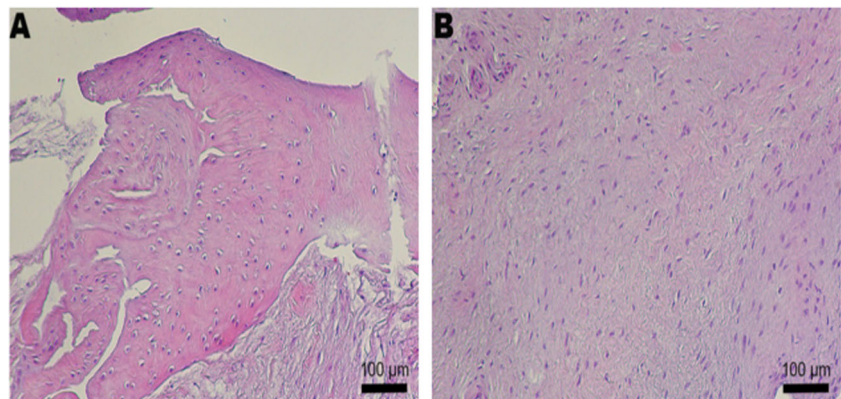
in meniscus cells of the fusiform (fibroblast-like cell type) and thus rich in type 1 collagens. Layer 2 resembled the middle red–white zone of the native human meniscus containing a mixture of oval and polygonal fibrochondroblast-like cells and fibroblast-like cells while being completely avascular. Layer 3 resembled the white or inner third zone with fibrochondroblast-like cells, characterised by its avascularity. It was concluded that the characteristic organisation seen suggested that ingrowth begins superficially and that a maturation process occurs in an inbound direction [20].

It is believed that CD34 staining is reflective of regenerative activity in the meniscus, and it was previously shown that three different cell types typically stain positively for CD34 [15]. These include fibroblastic cells at the height of the outer vascular zone of the meniscus, cells with round to oval morphology at the level of the superficial zone of the meniscus, and the endothelial lining of vascular structures. In our study, the greatest cellular and vascular CD34 staining intensity was seen in the viable allograft and polyurethane scaffold groups, possibly supporting increased regenerative activity. As previously demonstrated by Declercq et al., our study also found an absence of CD34 cells in the inner zone of the meniscus. The increased intensity of the CD34 staining within the viable allograft group versus the deep-frozen allografts may again reflect the treatment process undertaken

in the latter. However, the potential loss of cells within viable allograft types may be further augmented when more extended periods of time pass (e.g. due to a longer duration of testing or antimicrobial treatment). This may be an important consideration, as protocols for allograft treatment and processing evolve over time.

It was anticipated that α -SMA immunostaining would demonstrate the presence of contractile-cell phenotypes as part of the regenerative process in the specimens included. However, positive staining was only seen in the case of smooth muscle cells around blood vessels. The reason for this is unclear, as the positive control used in this study was also found to be working well. It may, however, relate to the period of time that elapses before biopsy if myofibroblastic cells are seen only during an earlier phase of the regenerative process. Declercq et al. previously reported no SMA-positive cells in their investigation of healthy menisci and menisci from patients with osteoarthritis [15]. Instead, they found that only biopsies from patients with a torn meniscus were positive for SMA, with the number of SMA-positive cells increasing in number as a function of time elapsed since the recognised date of meniscal injury. In that study, a biopsy was obtained between 1 week and 6 months. The generally longer time to biopsy and different injury and healing mechanisms may provide a reason for the different result seen in our study.

Fig. 6 Two different trends in morphology within the viable allograft group. Time to biopsy after implantation: **a** 32 months; **b** 24 months. Average cell density: **a** 567 ± 4.13 cells/mm²; **b** 900 ± 3.69 cells/mm²



In addition to cellular and vascular activity, the presence of ECM and collagen is also an important feature of meniscal tissue [21–26]. Aggrecan is the most common proteoglycan found in normal meniscal matrix and collagen type 1, the most common form of collagen. In our study, all biopsies obtained demonstrated a moderate or high level of both aggrecan and collagen, with no significant differences across groups. This data suggests that both allograft types and the polyurethane scaffold can facilitate ECM and collagen production.

In terms of study limitations, as only a small piece of the meniscus could be obtained from most patients, there is no way to be certain that the biopsy specimen was representative of the entire graft. This may also play a role in findings of SMA staining noted above. However, this is an inherent issue with all histological biopsy studies. Also, samples were obtained at different time points following implantation but with similar mean times across all groups. As approximately 20 specimens were obtained in all groups, and all were sliced extensively, it is believed that an accurate reflection of the findings from all three forms of meniscal material has been reported.

In conclusion, both viable allograft and a polyurethane meniscal scaffold show enhanced morphological, cell distribution and regenerative patterns over deep-frozen allograft following surgical implantation. Given the limitations in viable allograft availability, these findings support the continued development of synthetic scaffolds for meniscus replacement surgery.

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