New concepts of reconstructive techniques with human amniotic membrane in pelvic floor surgery

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Ph.D. Thesis

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List of full papers related to the subject of the thesis

I. **Barski D.,** Gerullis H., Ecke T., Varga G., Boros M., Pintelon I., Timmermans J.P., Winter A., Bagner J.W., Otto T. Repair of a vesico-vaginal fistula with amniotic membrane. Step 1 of the IDEAL recommendations of surgical innovation. Central Eur J Urol, 2015, 68(4):459-61. ***IF: 0***

II. **Barski D.,** Gerullis H., Ecke T., Yang J., Varga G., Boros M., Pintelon I., Timmermans J.P., Otto T. Bladder reconstruction with human amniotic membrane in a xenograft rat model: A preclinical study. Int J Med Sci, 2017,14:310-18**.** ***IF: 2.399***

III. **Barski D.,** Gerullis H., Ecke T., Varga G., Boros M., Pintelon I., Timmermans J.P., Otto T. Human amniotic membrane is not suitable for the grafting of colon lesions and prevention of adhesions in a xenograft rat model. Surg Innov, 2017, 24(4):313-20**.** ***IF: 1.909***

IV. **Barski D.,** Gerullis H., Ecke T., Kranz J., Schneidewind L., Leistner N., Queissert F., Mühlstädt S., Grabbert M., Tahbaz R., Pelzer A.E., Joukhadar R., Klinge U., Boros M., Bader W., Naumann G., Puppe F., Otto T. Registry of implants for the reconstruction of pelvic floor in males and females: A feasibility case series. Int J Surg, 2017, 42:27-33. ***IF: 2.211***

List of abstracts related to the subject of the thesis

1. Gerullis, H., Barski, D., Malmström, P.U., Sun, X., Ecke, T.H. Evidence in urologic- and pelvic-surgery research: Finding the IDEAL way of reporting. Biomed Res Int, 2017, 2716759. Editorial.
2. Barski D, Gerullis H, Winter A, Pintelon I, Timmermans JP, Ramon A, Boros M, Varga G, Otto T. Augmentation of rat bladder with human amniotic membrane graft. Eur Urology Supplements 2017, 15(3):e1027
3. Barski D, Gerullis H, Ecke T, Kranz, J., Schneidewind, L., Leistner, N., et al. Development of an online platform for registration and outcome measurement of urogynecological implants according to IDEAL-system. Int J Surg, 2016, 36, 141-42
4. Barski D, Gerullis H, Winter A, Pintelon I, Timmermans JP, Ramon A, Boros M, Varga G, Otto T. Reconstruction of bladder defects with amniotic membrane-IDEAL-D Stage 0-1. Int J Surg, 2016, 36(2), p 136
5. Barski D, Gerullis H, Varga G, Boros M, Pintelon I, Timmermans JP, Otto T. Reconstruction of rat bladder with human amniotic membrane graft. TERMIS, 2017, P394

INTRODUCTION

Due to increased life expectancy and optimized medical treatments, there is a clear need for novel, innovative approaches in reconstructive urology. The aim of tissue engineering (TE) reconstruction is to improve the postoperative functional outcome using customized natural materials to support the regenerative aspect of the healing process. Key factors which are identified for an optimal biological material are proper scaffold construction and proper regeneration of the urothelium and smooth muscle layers **[Drewa, 2015]**. The engineered scaffold aims to replace the extracellular matrix (ECM), support cell growth and expansion, and provide a mechanical and functional structure to replace and incorporate the native tissue. For long-term success, the scaffold should be resorbable or autologous to prevent foreign body reactions (FBR) that could lead to scarring **[Atala, 2012]**. However, the ideal matrix has not yet been identified, and it remains questionable whether there is a matrix that is suitable for the reconstruction of the entire urinary tract.

Another important question in TE reconstruction is why we have not seen more materials in clinical practice. The possible answer is that the main problem is the long transfer time and the diverse preclinical knowledge, starting with cell culture and *in vitro* studies, then *in vivo* animal models leading to clinical trials and commercialization, with regulatory oversight at all stages **[Ram-Liebig, 2015]**. The introduction of new surgical methods, innovations or variations does not yet follow clear standardized paradigms. **I**nnovation, **D**evelopment, **E**xploration, **A**ssessment and **L**ong-term Study (IDEAL) is a new reporting approach, introduced by an international panel of surgeons, researchers, editors, statisticians and other stakeholders who were committed to the production, dissemination and evaluation of quality research in surgery **[McCulloch, 2009]**. Recently, IDEAL stages have been adapted for devices (IDEAL-D) as well (see Table 1) to provide clear stages of surgical innovation which make it possible to assign all research to its particular level of development and evidence **[Sedrakyan, 2016]**.

Table 1. Stages of the IDEAL-D framework

		Primary outcome	**Study design**	**Patients**
Stage 0	Preclinical	Concept, safety	Experimental studies (animal, cadaver)	
Stage 1 **I**dea	"First in human"	Innovation	Case report, case series, registration	Single to few (<10)
Stage 2 **D**evelopment and **E**xploration	"Tinkering" with device, few adopters	Development, safety, efficacy	Prospective cohort trials	10–100
Stage 3 **A**ssessment	Stable procedure	Compare to standard, clinical outcome	Randomized controlled trial or similar	>100
Stage 4 **L**ong term	Registry, long-term evaluation	Quality assurance, identification of risk factors, comparators	Registry	>100

GOALS

The composition of the ECM of human amniotic membrane (HAM) is similar to that of the basement membrane in the urinary tract. Today, HAM allografts are used for reconstruction in eye and oral surgery and as dressings in burn patients. Therefore, our main goal was to prove that amniotic tissue can be an appropriate scaffold for reconstructive techniques in the urinary tract. We aimed to develop a standardized and manufacturer-independent process for amnion graft preparation, ensuring sterilization while keeping the ECM intact. Moreover, we aimed to ensure that the grafts provide feasible materials for surgical practice. Having achieved these goals, we conducted *in vivo* experiments, and, finally, we investigated the possibility of transferring experimental results into clinical practice following the steps in the IDEAL-D framework.

Study I. The main purpose was to develop an *in vitro* approach for amnion preparation and to preserve ECM integrity. Different mono- and multilayer techniques were tested to achieve a perfect hold and processing of HAM.

Study II. We aimed to investigate the possibility of bladder and colon grafting and the immunogenicity of processed HAM xenografts *in vivo*. Histological analyses were performed to investigate the degradation of HAM and graft rejection and the ingrowths of surrounding tissue 7, 21 and 42 days after the implantation.

The goal of **Study III** was to translate the *in vivo* animal results into a clinical scenario. HAM allografts were used to treat human vesico-vaginal fistulas and a case of a chronic wound with an entero-cutaneous fistula. The patients were followed up in a registry for one year.

MATERIALS AND METHODS

***In vitro* and *in vivo* experiments**

HAMs were obtained after elective Caesarean sections and informed consent from the patients. The amnion was detached from the chorion by mechanical traction, and the separated amniotic membranes were cut into 5x5 cm segments. The HAMs were fixed to a sterile silicon scaffold and frozen at -20°C for 24h until further use. (Pilot studies have shown that storage for up to six months does not cause degradation.) For further processing, the HAMs were thawed, sterilized in a 0.25% peracetic acid–alcohol mixture and incubated for 2h. Various HAM layers were tested to achieve the best processing with the possibility to cut and suture.

Fresh HAM (fHAM) and processed (frozen, sterilized and dried) HAM (pHAM) samples were sectioned and stained with H&E according to standard protocols for the *in vitro* studies. The tissues were stained for Ki-67 using rabbit primary antibodies to determine cell proliferation.

48 male Sprague Dawley rats were used at the Institute for Surgical Research of the University of Szeged, Hungary (license number V./146/2013) for the *in vivo* experiments. The rats were anaesthetized with 40 mg/kg 10% ketamine, a midline laparotomy was performed, and the bladder pressure was evaluated with the cystometric method developed by Lundbeck et al. Subsequently, a defined 0.5 cm lesion was cut at the bladder dome. In the second group, a defined 0.5 cm length of the caecum wall was resected. In the treated amnion groups (bladder n=18 and colon n=18), a multilayer amnion patch was trimmed to cover the defect size (10x10 mm) and was fixed to the bladder or colon wall with 6-0 Monocryl single sutures at 3–4 points. Additional human fibrin glue was used to seal the lesion. In the first control group (B1, n=6 and C1, n=6), the defect was closed with a single Monocryl 6-0 running suture and fibrin glue. In the second control group (B2, n=3 and C2, n=3), the amnion graft was sutured to the bladder or colon wall without a prior lesion. The animals were sacrificed at one week (Ba and Ca, n=10; B1 and C1, n=4; B2 and C2, n=2), three weeks (Ba and Ca, n=12; B1 and C1, n=4; B2 and C2, n=2) and six weeks (Ba and Ca, n=10; B1 and C1, n=4; B2 and C2, n=2) after surgery. Bladder

capacity was determined again, and tissue samples (urinary bladder or colon, kidneys and spleen) were collected. HAM grafts, colon and bladder wall reactions were assessed, abscess and adhesion formation was recorded, and the area was graded semiquantitatively between 0 and 3 according to the van der Ham score **[van der Ham, 1992]**. Deparaffinized sections (5 µm) were used for histology, and the tissue architecture and cell infiltration were visualized with H&E staining. For immunohistochemistry, tissue sections were deparaffinized, rehydrated and subjected to a heat-induced antigen retrieval (citrate buffer, pH 6.0), followed by 3% hydrogen peroxide and avidin-biotin blocking. Prior to incubation with the primary antiserum, sections were incubated with a PBS blocking solution containing 10% normal horse serum, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN_3 and 1% Triton X-100. Primary and secondary antisera were diluted in a blocking solution without Triton X-100. Sections were incubated overnight with the primary antibody and immunostained with the streptavidin–biotin peroxidase method, followed by a diaminobenzidine (DAB) chromogen solution. Finally, sections were counterstained with haematoxylin. Negative controls were incubated in a blocking solution without primary antibodies. Digital images of H&E and α-actin were used for the evaluation of smooth muscle content within the reconstructed wall. Pictures were taken using a Zeiss Axiophot microscope equipped with an Olympus DP70 digital camera at 4x magnification. HAM thickness was measured (in µm), with particular attention paid to the transition zone between the amnion graft and the normal bladder or colon wall. A semiquantitative score from 0 to 3 for inflammation and from 0 to 2 for vascularization was used. Inflammation of the implant region was scored by counting lymphocytes in ten fields of 0.25 mm^2 in three observer-randomized H&E-stained slides (0= <5% cells/field; 1= 5–25%; 2= 25-50%; 3= >50%; 200x magnification). A similar score was used for vascularization (0= 0 vessels/mm^2, 1= 1-3 v/mm^2; 2= >3v/mm^2; 200x magnification). Kidney and spleen specimens were analysed for signs of transplant rejection.

Clinical studies

I. A 64-year-old female was admitted to the Department of Urology, Lukas Hospital Neuss, Germany, with sigma diverticulitis and a chronic vesico-vaginal fistula (VVF). She had a previous history of cervical cancer and radio-chemotherapy for anal cancer with complete remission. The VVF persisted after multiple abdominal operations, and the patient suffered from permanent incontinence and a local skin

infection. After a recovery period of three months, we re-evaluated the patient with cystography, cystoscopy, ureteropyelography and a vaginal examination. A complex vesico-vaginal fistula of 1.5 cm was detected at the apical anterior vaginal wall leading to the bladder base. Additionally, scarring of the vagina and distal ureters was found, and the patient suffered from a persistent MRSA colonization of the urine. Due to the complex situation, it was decided that an individualized treatment would be used with off-label use of HAM. Prior to surgery, three layers of HAM were fixed with a 4-0 Monocryl suture, and a multilayer HAM of 4 cm was used as a graft to close the defect at the edges of the normal bladder wall.

II. An 83-year-old female underwent a radical cystectomy with a cutaneous ureterostomy for locally invasive bladder cancer at the Department of Urology, Lukas Hospital Neuss, Germany. Two weeks after surgery, she was admitted again with a severe infection of the laparotomy wound. A wide resection of subcutaneous tissue with vacuum sealing was necessary, followed by displacement plastic skin repair. Seven days later, the patient presented with a cutaneous fistula with purulent secretion and communication with a small bowel loop. Two layers of amnion dressing were applied weekly after cleaning and debridement for four weeks. The wound size was documented, and a biopsy at the wound margin was performed at week four.

Statistics

Data from different experimental groups were compared using the Mann-Whitney U test and the Kruskal-Wallis test. Data are presented as medians with ranges or means with standard deviation (SD). P values <0.05 were considered significant.

RESULTS

In vitro experiments

Processed pHAMs were compared with fresh tissues by histology using H&E staining. pHAM maintained tissue thickness and structural integrity on a comparable level with the amniotic layer of fresh tissue. However, neither fresh amnion nor pHAM displayed any positive staining for Ki-67. For the best hold, 4-0, 5-0 and 6-0 Monocryl sutures were used at 3-4 points to fix the HAM to the scaffold.

In vivo studies – bladder reconstruction with amnion xenografts

Two animals (11%) died in the treated group: one due to postoperative sepsis, another during anaesthesia. No animals were lost from the control groups. No other complications higher than grade II (Clavien–Dindo classification) were observed

[Clavien, 2009]. The bladder capacity did not change in the treated group but decreased significantly in control group B1 with the suture of the lesion ($p=0.01$).

No signs of severe inflammation were found in the abdominal cavity during reoperation. Meso-adhesions to the HAM graft were detected in most of the treated cases. HAM appeared as a thick, oedematous graft with inflammation running from the middle towards the transition zone of the bladder wall at day 7. At days 21 and 42, HAM was still well defined, albeit with reduced inflammation. Adhesions were present in some cases. Inflammation was less prominent in the control groups.

The xenotransplanted HAM graft covered the bladder wall and maintained its architecture at day 7. The lesions could be recognized as regions without smooth muscle cells but with abundant connective tissue and signs of inflammation. Significant inflammation and an increased number of blood vessels were observed in the amnion and between the amnion and the adventitia of the bladder, resulting in an enlarged amnion. Infiltrated lymphocytes agglomerated mostly in the area bordering the HAM. At day 21, the amnion appeared less thick, and the inflammation was significantly diminished ($p<0.05$). New capillaries started to grow into the surrounding connective tissues, and scattered smooth muscle cells emerged in the area of the lesion. In the control groups, signs of inflammation had mostly disappeared. At day 42, it became more difficult to discern the region of the lesion, where the amnion formed a thinner layer on the bladder wall with no signs of degradation. Inflammation was markedly reduced in the amnion and in the zone between the amnion and the bladder wall ($p<0.05$). The number of large vessels in the amnion appeared to be reduced and periamniotic vascularization increased, but these results were not significant. Connective tissue, bundles and thin muscle layers were found in abundance in all groups. Smooth muscle regeneration occurred more rapidly in the amnion group, although the difference in the regeneration compared to the control group with the suture of the lesion was not significant ($p<0.05$).

***In vivo* colon reconstruction with amnion xenograft**

During reoperation, the HAM appeared as a thick oedematous graft with signs of inflammation, but severe inflammation was not found in the abdominal cavity. The inflammation was less severe in the control groups. Strong adhesions of the HAM graft to the small bowel and abdominal wall that withstood tractions were detected in most of the treated cases. At day 7, a higher adhesion score with a larger coverage area was found in the amnion group (1.8 ± 0.45) vs. group C1 (0.5 ± 0.7) ($p<0.05$).

However, similar adhesion scores were present in groups C1 and C2. At day 21, the adhesion score was higher in the amnion group (1.8±0.84) vs. group C1 (1±0) ($p=0.178$), but the difference was statistically not significant. HAM could not be identified and in some cases could not be detected at day 42. The adhesions increased in the amnion group vs. groups C1 and C2 ($p=0.052$).

At day 7, strong inflammation with abundantly increased numbers of lymphocytes and blood vessels was observed in the amnion layers and between the amnion and the submucosa of the colon, which resulted in an enlarged amnion. A lower-level, but still strong inflammation was found in control groups C1 and C2. HAM thickness was reduced, and the inflammation was significantly decreased ($p<0.05$) at days 21 and 42. Connective tissue bundles and scattered smooth muscle cells appeared in the area of the lesion. In control group C1, signs of inflammation (the presence of lymphocytes) had mostly disappeared, and there were also no clear signs of regeneration of smooth cells in the lesion region. At day 42, it became more difficult to verify the presence of the amnion in the treated group, and the different layers of the amnion could no longer be distinguished. The amnion, which was significantly reduced in thickness, formed a thinner layer on the colon wall. In the control group without lesions (C2), the amnion was completely degraded and could no longer be detected. Inflammation was markedly reduced in the amnion and in the zone between the amnion and the colon wall, but it was still significantly higher compared to control group C1 ($p<0.05$). Despite the presence of scattered smooth muscle cells and bundles, it was difficult to measure if there was a clear regeneration of smooth muscle compared to control group C1.

No macroscopic signs of rejection were found in the kidney and spleen specimens. However, four out of six animals in the treated group showed an affected kidney at day 21. To rule out an obstruction or increased bladder pressure as the cause of these findings, we compared the results with the colon study, and the same results were demonstrated. The changes were subtle with slightly enlarged tubuli and a slightly enlarged urinary space, the glomeruli appeared denser, and no presence of immune cells or other signs of transplant rejection were found. At day 42, the kidneys of two out of five animals were still slightly affected, whereas no such changes were detected in the controls. On the whole, this suggests that a transient, subtle transplant glomerulitis was present in the rats with HAM grafts.

Clinical cases - vesico-vaginal fistula

Seven days after surgery, a cystography was performed, and no leakage was present. In the next three months, re-evaluations with cystography, cystoscopy and a vaginal examination were performed, no recurrent fistula was found, and full recovery of the vaginal epithelium was present. The ureter stents were internalized, and the patient was able to micturate without incontinence. The bladder capacity was restored to a volume of 250 ml. No severe complications and no signs of graft rejection were observed over a period of six months. The MRSA colonization in the urine was successfully eradicated. However, during the follow-up at ten months, the patient reported vaginal urine leakage again and a recurrent fistula was observed at the margin of the amnion graft.

Treatment of an entero-cutaneous fistula

The wound size was 3x2x1 cm when the HAM was applied. After two weeks, the secretion stopped, and the ulcer was reduced by 20% and then healed after eight weeks with four applications of HAM. The healing was complete after six-month and nine-month follow-ups. Histological analysis showed re-epithelialization and recovery of muscle cells four weeks after the treatment. The HAM did not degrade and could still be detected as a smooth layer on the surface. Moderate inflammation was present, but signs of graft rejection were not observed.

DISCUSSION

There are several methods for amnion preparation, but none of them has been compared properly or standardized. For clinical use, HAMs should be intact and free from contamination. HAM is sterile when collected during a Caesarean section **[Adds, 2001]**, but sterilization is still recommended to avoid the risk of infection. Cryopreserved, dried, irradiated or freeze-dried amniotic tissues have already been used in previous studies. In our experiments, amniotic membranes were stored at -20°C with PBS to produce a safe, effective and minimally manipulated tissue. A 0.25% peracetic acid and ethanol mixture was used for sterilization, a procedure proven to be reliable for preserving ECM and accepted by the Paul Ehrlich Institute **[Pruss, 2001]**. HAM is fragile and must be handled with care to avoid tearing and rolling, so for this reason, dried HAMs were fixed by suture to a sterile silicon scaffold. Next, we used several layers of HAM to provide better handling and more stability for the next steps of the planned tissue reconstruction.

Urinary tract reconstruction with amniotic tissue

HAMs were used in urology in 1955 for the reconstruction of the urethra **[Lenko, 1955]**. However, only a few successful attempts have been reported to date. Long ureteral wall strictures (5.5 cm) were supplemented by using folded HAM allografts, and good results were reported after an average follow-up period of 25.2 months **[Koziak, 2007]**. Brandt and his colleagues successfully reconstructed a female urethra using autologous grafts prepared from HAM **[Brandt, 2000]**. Adamowicz et al. designed a sandwich-structured biocomposite material from a frozen cell-seeded (i.e. bone marrow-derived mesenchymal stem cells) membrane covered on both sides with two-layered membranes prepared from electrospun poly-(L-lactide-co-ε-caprolactone) (PLCL). The authors considered this reinforcement of the AM necessary because of its poor mechanical qualities. The new biomaterial (10x10 mm) was used for bladder augmentation after hemicystectomy in rats **[Adamowicz, 2016]**. Immunohistochemical analysis showed effective regeneration of the urothelial and smooth muscle cells and complete PLCL degradation. However, the authors reported a moderate inflammatory reaction after three months. Our results confirmed these previous reports. In our study, no signs of leakage or unchanged bladder capacity were observed after reconstruction. The inflammatory reaction had already almost disappeared after six weeks. It should be added that larger grafts require sufficient nutrition of the cells and removal of waste products to eliminate/reduce the risk of fibrosis and shrinkage. With a diffusion distance from the supplying blood vessel of ~150–200 µm, HAMs efficiently conduct sufficient oxygenation **[Laschke, 2006]**. HAM also acts as a basal membrane and thus facilitates the migration and enhanced adhesion of the epithelial cells **[Sonnenberg, 1991]**. Although we expected a faster regeneration of smooth muscles, the regeneration could not be evaluated in a standardized way due to the smaller size of the grafts and an overlap with the normal bladder wall. Nevertheless, no signs of graft shrinkage or necrosis were found over a six-week period. We had success using the unseeded amniotic membranes in a three- to four-layer technique. Incorporation of autologous urothelial and smooth muscle cells involves a costly and time-consuming process of cell harvesting, culturing and seeding. Furthermore, cells from diseased bladders may behave differently from normal cells, rendering their use for tissue engineering applications questionable **[Roelofs, 2016]**. In addition, there are some data that show that scaffolds without seeded urothelial cells perform better than those with seeded cells **[Engel, 2014]**. The

limitation of our study is the relatively small size of the grafts (10x10 mm) and the relatively short follow-up period. The process of regeneration in larger constructs and damaged tissue conditions also needs to be evaluated.

Bowel tract reconstruction with the amniotic tissue

Adhesions are key problems in the peritoneal cavity following surgeries **[Brochhausen, 2011]**. Several strategies have been proposed for the prevention of adhesion formation, including the use of allogenic (e.g. peritoneum), xenologous materials (e.g. collagen), synthetic materials (e.g. polymers) and sealants (fibrin), but the optimal method has not yet been identified and translated into clinical practice. We aimed to prove that HAM can prevent adhesion formation in bowel surgery. For this purpose, we designed a rodent model of anastomosis leakage and repair of small defects in the caecum. The results demonstrated that the use of amniotic membranes for the repair of bowel lesions is not beneficial in a xenograft model as compared to a standard therapy. We did not find clinical signs of rejection, but we did observe adhesions and the presence of inflammatory reaction in the HAM-treated group. (89% in the treated group vs. 33% in the control group, $p<0.05$). Moreover, the severity of inflammatory reactions was significantly higher in the amnion-treated group, probably due to insufficient bowel sealing with the graft. Similarly, an increased number of inflammatory cells was reported by other groups after the application of HAM in rodent models **[Uludag, 2009]**.

Only a few reports are available to date on the use of HAMs in reconstructive bowel surgery **[Schimidt, 2010]**. Several studies have shown the degradation of the ECM components from bacterial-derived metalloproteases in the colon **[Pasternak, 2010; Shogan, 2015]**. If applied as a graft, a rapid degradation of the amnion occurs between days 14 and 90, depending on the grafted tissue and number of layers **[Kesting, 2008; Schimidt, 2010]**. In our study, the transformation of multilayer HAMs, tissue reorganization and degradation were observed between days 21 and 42. We used fibrin glue as well, though no effects of fibrin glue on adhesion formation have previously been reported **[Kanellos, 2006]**. Another limitation of our model was the healthy animal population. A peritonitis model, as proposed by several groups, could be more appropriate to clarify the protective role of HAM on the anastomosis line **[Wichtermann, 1980]**. In a rat model of colon anastomosis where the caecum was covered with a 10 mm amnion wrap, the leakage rate was as high as 25% after seven days in the standard anastomosis group vs. 0% in the amnion

group **[Uludag, 2009]**. Unlike in our model, the authors reported a high dehiscence rate of 40–50% in the control group with standard anastomosis. The difference is probably due to the different experimental design. In our study, no signs of dehiscence were detected in the control group with the suture of a small bowel wall defect. However, the increased initial inflammation and adhesions in the amnion group appear to be a sign of insufficient bowel sealing with an amnion graft. This notion is supported by the finding that one case of postoperative peritonitis and sepsis was detected in the HAM group.

In conclusion, we suggest that HAMs alone are not suitable barriers to preventing adhesions and inflammation, and therefore this technique is not useful or superior to a standard suture for the reconstruction of the bowel wall in a xenograft model. However, the anastomosis-protective potential of HAM use in a high-risk situation (e.g. peritonitis model) needs to be evaluated further. Other limitations of our study were the rather small size of the grafts (10x10 mm), the short follow-up, the small sample size and the xenograft model itself. Nevertheless, it should be added that a smaller number of elements was purposefully planned as is the case with proof-of-principle studies.

The xenograft animal model and the IDEAL recommendations

Rodents are employed as standard cost-effective models for most preclinical trials. We first conducted animal experiments to clarify the possibility of graft rejection in a xenograft rodent model and the applicability of HAM as a future biomaterial in accordance with the IDEAL-D recommendations. No clinical signs of rejection were detected, although a transient inflammatory reaction was seen in the amniotic and periamniotic tissue. A similar transient increase in inflammatory cells was reported in the case of cryopreserved HAMs for soft tissue repair in rats **[Kesting, 2008]**. Additionally, most animals in the amnion group presented a subtle acellular transplant glomerulitis. These changes can be classified as borderline mild acute rejection according to the Banff classification **[Bhowmik, 2010]**. It is likely that the glomerulitis was due to the xenologous model and would not develop in an allograft. This notion is supported by the fact that HAMs in human patients did not induce inflammation and graft rejection; the grafts were well-tolerated without side-effects. Nevertheless, future studies should test different HAM-grafting techniques in animal models of peritonitis and fistulas to simulate the outcome in humans.

HAM-assisted repair of a vesico-vaginal fistula

Complex vesico-vaginal fistulas usually present a poor outcome with high recurrence rates and a devastating clinical course. Radiation-induced recurrent vesico-vaginal fistulas have the lowest success rate and require the most demanding interventions **[Ghoniem, 2014]**. Vascularization of the graft or flap is the main requirement for successful healing. In our case, HAM was used as a sealing onlay graft and only served as a transient seal in a damaged radiated tissue in a patient with a recurrent fistula ten months after the initial surgery. There is only one published report on VVF repair with an amniotic membrane allograft in an irradiated field with a robotic procedure **[Price, 2016]**. The vaginal and bladder fistula defects were closed separately in two layers using interrupted 4-0 polydixanone sutures. A rehydrated amniotic membrane patch was used as an interposition graft between the vaginal wall and bladder and was sutured in place over the surface of the vaginal side. However, the follow-up was shorter, only five months. In addition, the processing of the amnion allograft was different **[Koob, 2014]**. A potential problem with the dehydrated allograft is the unknown duration of the angiogenic effect, and thus patients with radiation fistulas remain at risk for recurrent fistulas for years **[Price, 2016]**. Further, well-documented clinical trials are needed that share experience with the technique and handling HAM to shed light on the potential value of HAM for the treatment of VVF. Due to the rare condition, it is important that every case should be registered and reported (IDEAL Stage 1).

HAM dressing for the treatment of chronic wounds

Amnion can act as a physical barrier against bacterial contamination and can also create a moist environment for healing **[Mao, 2016]**. Dehydrated amniotic membrane and chorion (dHACM) have been used in randomized trials for the treatment of chronic diabetic foot ulcera (DFU) **[Laurent, 2017]**. In a prospective, randomized, single-centre clinical trial, wound reduction and rates of complete healing were investigated in patients with DFU treated with dHACM versus standard care, and significant differences were observed in wound reduction at four and six weeks **[Zelen, 2013]**. Another prospective randomized trial of 40 patients with chronic DFUs reported an average time of 2.4 weeks until complete healing **[Zelen, 2014]**. However, most of the studies analysed the use of HAM and HACM in clinically uninfected cases. Only one randomized study used a tissue-engineered form of wound dressing containing acellular human amniotic collagen membrane vs. wet

dressing in patients with partially infected wounds. The complete healing rate was significantly higher for the amnion group (40.7% vs. control group, 16.7%) **[Mohajeri-Tehrani, 2016]**. We were the first to report on the successful treatment of an entero-cutaneous fistula and infected wound with HAM. In our case, the external dressings were changed every two days due to the initial infection of the wound. HAM is simple to use and makes it possible to cover larger and irregular wound areas as well as deep tunnelling wounds. Future studies in larger numbers of patients with infected wounds should be conducted, and inclusion of amnion dressings in the algorithm of secondary wound care should also be evaluated. However, the results suggest that HAM can be a cost-effective treatment for chronic wounds and can reduce hospitalization time.

The project with regard to the IDEAL-D recommendations

The protection of intellectual property should be regulated by the authorities at the early stages of development **[Sedrakyan, 2016]**. Animal experiments represent safe opportunities to optimize reconstructive techniques with HAM, but results from animal or cadaveric studies cannot be directly translated to human studies and should be considered as preliminary models for the biological performance of implant materials (i.e. IDEAL-D Stage 0). In our case, a functionally useful description of the use of HAMs was presented for the repair of VVF and entero-cutaneous fistulas (i.e. IDEAL-D Stage 1). This procedure should prevent surgeons and researchers from repeating a harmful error reported by another investigator and bundling the knowledge. With the next step, prospective and quasi-randomized clinical development studies (Stage 2) with the application of HAM for ureteral reconstruction, protection of the urethra anastomosis and repair of fistula conditions in the urinary tract can be planned. These studies will be able to describe the technical modifications in surgical procedures and establish a consensus among surgical teams ($n<100$). At this stage, the technique is performed by the investigator and a few other innovators, with approval by regulatory authorities as the aim. The main outcome criteria and complications should be identified and reported using a validated classification. If possible, randomized trials are required at IDEAL Stage 3. Data should be included in a registry at an early stage to uniquely identify the patient, to enable surgeons to track outcomes and adverse events and to vet findings from smaller observational studies (Stages 2–4). Our research group used an international registry and our own web-based one **[Agha, 2017; Barski, Int J Surg**

2017]. The main problem at this stage is the challenging technical requirements for producing TE materials. Manufacturing sites inspected according to the principles of Good Manufacturing Practice are required. Such issues become even more challenging when seeded matrices are used. In Europe, the process is constrained further by regulatory challenges because of legislation in individual countries within the EU that are regulated by the European Medicines Agency **[Ram-Liebig, 2015; Sievert, 2017]**. We need a safe regulatory system that does not hinder innovation unnecessarily and encourages the production of adequate valid evidence of safety and efficacy. IDEAL-D could provide a framework for the regulation of TE studies and innovation in the future.

SUMMARY OF THE NEW FINDINGS

The development of this surgical device (i.e. the use of HAM in reconstructive urology) strictly followed the IDEAL-D recommendations at every step to ensure comparability and transparency.

1. A sterilization process was developed for HAM, and a method was designed to achieve stability and better handling during surgery. Several layers of HAM were attached to a silicon scaffold, and histological evidence was provided that the ECM structure and collagen composition were preserved during the processing method.

2. Unseeded HAM grafts were used for the *in vivo* reconstruction of the urinary bladder in a short-term xenograft study. No signs of graft shrinkage or necrosis were demonstrated during a six-week follow-up period and only a minimal immunological reaction developed. The functional and histological results demonstrated the suitability of HAM for the reconstruction of the rat urinary tract.

3. The unseeded HAMs increased the adhesion formation and were not suitable for the repair of bowel defects in rats. HAM grafts are not indicated for the reconstruction of the bowel wall. The anastomosis-protective property of HAM in high-risk situations (e.g. peritonitis models) needs further evaluation.

4. We demonstrated the possibilities for the transient use of HAM in clinical practice as sealing onlay grafts in a complex vesico-vaginal fistula case.

5. We demonstrated that an entero-cutaneous fistula with an infected wound can be successfully treated with several HAM dressings. The use of HAM allowed for the coverage of large and irregular surface areas and deep tunnelling wounds. This suggests that HAM can be a useful, potential scaffold for reconstruction surgeries.

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