Creation of Abdominal Aortic Aneurysms in Sheep by Extrapolation of Rodent Models: Is It Feasible?

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Background: Abdominal aortic aneurysms (AAAs) are a potentially deathly disease, needing surgical or endovascular treatment. To evaluate potentially new diagnostic tools and treatments, a large animal model, which resembles not only the morphological characteristics but also the pathophysiological background, would be useful.

Methods: Rodent animal aneurysm models were extrapolated to sheep. Four groups were created: intraluminal infusion with an elastase-collagenase solution ($n = 4$), infusion with elastase-collagenase solution combined with proximal stenosis ($n = 7$), aortic xenograft ($n = 3$), and elastase-collagenase–treated xenograft ($n = 4$). At fixed time intervals (6, 12, and 24 weeks), computer tomography and autopsy with histological evaluation were performed.

Results: The described models had a high perioperative mortality (45%), due to acute aortic thrombosis or fatale hemorrhage. A maximum aortic diameter increase of 30% was obtained in the protease-stenosis group. In the protease-treated groups, some histological features of human AAAs, such as inflammation, thinning of the media, and loss of elastin could be reproduced. In the xenotransplant groups, a pronounced inflammatory reaction was visible at the start. In all models, inflammation decreased and fibrosis occurred at long follow-up, 24 weeks postoperatively.

Conclusions: None of the extrapolated small animal aneurysm models could produce an AAA in sheep with similar morphological features as the human disease. Some histological findings of human surgical specimens could be reproduced in the elastase-collagenase–treated groups. Long-term histological evaluation indicated stabilization and healing of the aortic wall months after the initial stimulus.

INTRODUCTION

To study new potential surgical and endovascular therapies of abdominal aortic aneurysms (AAAs), there is a need for large animal models. For the evaluation of technical feasibility and hemodynamic effects of endovascular treatments, patch techniques are used, using different types of material including peritoneum, jejunum, veins, polytetrafluoroethylene, and so forth[1–4] Another technique to obtain a mechanical equivalent of aortic dilatation is endovascular dilatation of the aortic wall followed by stent placement.[5] The biological and histological similarities of these

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models with human AAA are sparse. Not only the evaluation of the technical feasibility of new treatments is important but also the impact on disease pathophysiology needs to be studied. Therefore, these animal aneurysm models should also mimic pathophysiological features of human aneurysms, such as atherosclerosis, presence of intramural thrombus, cracks in the elastic layer, inflammatory infiltrate within the adventitial and medial layers, and increased proteolytic activity within the aneurysm wall. Several small animal aneurysm models with these features are available and have been an integral part in the development of drug therapies.[6–8] The most frequent chemically induced method within rodents is an elastase-induced model.[8] Proteases induce an immune-mediated elastin destruction leading to the formation of aneurysms.[8,9] Another frequently used AAA model in mice is angiotensin II (Ang II) infusion.[10] Aneurysms develop in the suprarenal aorta following Ang II infusion with a subcutaneous osmotic pump in hyperlipidemic mice.[11,12] The xenograft model uses transplantation of the infrarenal aorta from one species to another, for example, guinea pig to rat.[13] Before implantation, the graft is decellularized using sodium dodecyl sulfate (SDS). This decellularization of the donor tissue triggers a slower interspecies immune response and not an acute rejection.[12,14] Because of their small-diameter vessels, these rodent models are not appropriate for device testing. In larger animal species, spontaneous aortic aneurysms are rare but occur in horse lineages,[15] although these are difficult to work within experimental conditions. Several groups evaluated protease infusion or xenograft transplantation in dogs and pigs; an overview is depicted in Table I. The obtained diameter increases were quite diverse and difficult to reproduce. Several suggestions were made to improve the results, such as combining elastase with collagenase or placement of a stenosing cuff in the subrenal aorta causing alterations in blood flow.[17,18,20–23] For ethical reasons and differences in the coagulation system with humans, dogs are difficult to work with. Sheep have characteristics that make them more attractive for cardiovascular research than pigs: their easy handling, stable weight, and coagulation system similar to that of humans.[25] Therefore, the goal of this study was to extrapolate the protease infusion and xenograft implantation model to sheep and perform long-term follow-up.

MATERIAL AND METHODS

Operation

The Ethical Committee for animal experiments at the KU Leuven approved the experiment (number P144-2010). Sheep (female, 1-year-old) were sedated with intramuscular injection of ketamine (15 mg/kg). Anesthesia was induced with isoflurane 5%, the animals were intubated, and an oral-gastric tube was placed. Isoflurane (2–4%) maintained anesthesia. An intravenous line and arterial line were inserted. Heart rate, blood pressure, end-tidal CO_2, and blood O_2 saturation were constantly monitored. Through a retroperitoneal incision, the abdominal aorta was exposed. After administration of heparin (5000 U), a shunt was placed from the descending aorta to the aortic bifurcation, supplying blood to the spine and lower limbs during aortic clamping (Fig. 1). Then, the aorta was clamped proximally (below the renal arteries) and distally (above the bifurcation), as well as the lumbar arteries. Through a catheter, the aortic segment was infused with an elastase (67.2 U, 11.2 U/mL, lot 041M7018V, E-1250, Sigma-Aldrich)—collagenase (750 U, 125 U/mL, lot 010M8623 − 011M8616V, C9891, Sigma-Aldrich) solution (pH: 8.5) for 30 min at 100 mm Hg (groups A and B).[19] In group B, an additional proximal stenosis was added by ligature of the subrenal aorta around an 11-French sheath after protease infusion. In group C, the aorta was replaced with an SDS (L-3771, Sigma-Aldrich) treated porcine xenograft. After explantation, the pigs' aorta was rinsed with 0.9% NaCl solution and transferred to a 0.1% SDS solution at 37°C for 48 hr.[14] Thereafter, the sample was rinsed once again and stored in sterile saline solution at 4°C for a maximum of 3 days. Microscopically, this led to a complete decellularization with an intact scaffold (Fig. 2). In group D, the aorta was replaced with an SDS and protease-treated xenograft. The protease luminal perfusion was done ex vivo just before implantation, 30 min at 100 mm Hg with identical doses as groups A and B. At the end of the operation, the wound was closed in layers, and the animal was extubated. The first 10 days postoperatively, the sheep received low-molecular-weight heparin (enoxaparin 20 mg/day). The health status of the animals was checked on a regular basis.

Computed Tomography

To evaluate the baseline diameter, computed tomography (CT) was performed before surgery (groups A and B) or on the day after surgery (groups

Table I. Overview of protease and xenograft models in large animals

		Model										
		Protease model										
Study	Animal	Elastase	Collagenase	Balloon dilatation	Other	Duration infusion	Amount elastase (U)	Amount collagenase (U)	Animal number (intervention)	Follow-up (weeks)	Diameter increase	Specific histologic changes
Boudghène, 1993[16]	Beagle dogs	Yes				40 min	2,800		8	3	66%	- Elastolyse media - Medial thinning
Strindberg, 1998[17]	Mongrel dogs	(1-2-3)	(3)	(1) Yes (2) No (3) No		(1) 40 min (2) 2 hr (3) 2 hr	(1) 2,800 (2) 2,800—8,400 (3) 3,400—8,400	(1) - (2) - (3) 1,800—3,200	(1) 3 (2) 5 (3) 2	(1) 7—29 (2) 4 (3) 0—4	(1) No (2-3) 66%	(1-2-3) - Mural thrombi - Intimal hyperplastic reaction - Elastolyse media - SMC reduction (1) - Fibrotic collagenous capsule adventitia (3) - Inflammatory infiltration adventitia
Marinov; 1997[18]	Yucatan miniature swine	Yes				Unknown	300—938		6	4 days to 3 weeks	No	- Intimal hyperplastic reaction - Elastolyse media - Medial thinning - Inflammatory infiltration - SMC reduction - Calcium deposits
Hynecek, 2007[19]	Yorkshire swine	Yes	Yes	Yes		20 min	30	8,000	10	24 hr to 6 weeks	94 ± 37%	- Partial endothelial loss, followed by re-endothelialization - Inflammatory infiltration - SMC disorganization - Elastin disruption and collagen deposition

(*Continued*)

Table I. Continued

		Model										
		Protease model										
Study	Animal	Elastase	Collagenase	Balloon dilatation	Other	Duration infusion	Amount elastase (U)	Amount collagenase (U)	Animal number (intervention)	Follow-up (weeks)	Diameter increase	Specific histologic changes
Sadek, 2008[20]	Yorkshire swine	Yes	Yes	Yes		20 min	1,000	16,000	14	1−4	Unknown	Unknown
Molacek, 2009[21]	Pigs	Yes			Subrenal stenosis	30 min	429		7	3	113%	- Thickening of the intima - Elastolyse media - Inflammatory infiltration media and adventitia
Czerski, 2013[22]	Pigs	(1-2-3)	(2-3)	(1) Yes (2) Yes (3) Yes	(3): CaCl	(1) 20 min	(1) 500 (2) 500 (3) 500	(1) - (2) 6,000 (3) 6,000	(1) 4 (2) 4 (3) 4	4	(1) 37.4 ± 8.6% (2) 76.6 ± 9.3% (3) 104.2 ± 11.3%	(1) - Calcium deposition media and adventitia - Inflammatory infiltration adventitia (2) - Pronounced calcium deposition media and adventitia - Inflammatory infiltration adventitia - Elastolyse media - SMC reduction (3) - Massive calcium deposits - Elastolyse media - SMC reduction - Pronounced inflammation adventitia

Kloster, 2015[23]	Danish Landrace pigs	Yes	Yes	Subrenal stenosis	30 min	Unknown	10	4	57 ± 10.2%	- Preserved endothelium - Elastolyse media - Moderate SMC atrophy - Unaffected adventitia
Riber 2017[24]	Pigs	Xenograft model (sheep to pig)					6	7	81 ± 30.20%	- Neointimal hyperplasia - Destruction of media and elastolyse - Adventitial thickening - Inflammatory infiltration adventitia

(1-2-3), subgroups; SMCs, smooth muscle cells.

Fig. 1. Peroperative picture of xenotransplant (*arrows*) with passive shunt in place (*).

C and D). Six, 12, and 24 weeks after operation, CT was repeated. Therefore, the sheep was sedated with intramuscular injection of ketamine, and anaesthesia was performed using isoflurane (2—4%). Two independent observers (M.C., D.V.), blinded for the treatment group, measured the maximum diameter in anteroposterior direction of the infrarenal aorta using OsiriX software (v3.8.1, Geneva, Switzerland). Results are depicted as the relative diameter change (in percent), which is the measured diameter minus the baseline diameter, divided by the baseline diameter.

Microscopic Evaluation

Autopsy was performed 6, 12, or 24 weeks after operation. The intention was to obtain at least 1 animal from each group at each time point. The date of sacrifice of each individual animal was defined at the initial operation. In case of early mortality, a full autopsy was performed to define the cause of death. Briefly, heparin was administered, and the animal was euthanized using high-dose pentobarbital and potassium. The aortic and surrounding tissues were explanted. Slides were stained with hematoxylin and eosin and Van Gieson's stain. In case of calcium deposition, additional Von Kossa staining was performed. An experienced pathologist (E.V.), blinded for the sample group, performed a qualitative evaluation of the architecture and inflammatory response on 3 cross sections per staining and a magnification of 12.5, 50, and 200×.

RESULTS

Operation

Preliminary experiments ($n = 2$) showed the occurrence of paraplegia directly after the operation. Therefore, the protocol was adapted, and a bypass was created during the operation to obtain adequate

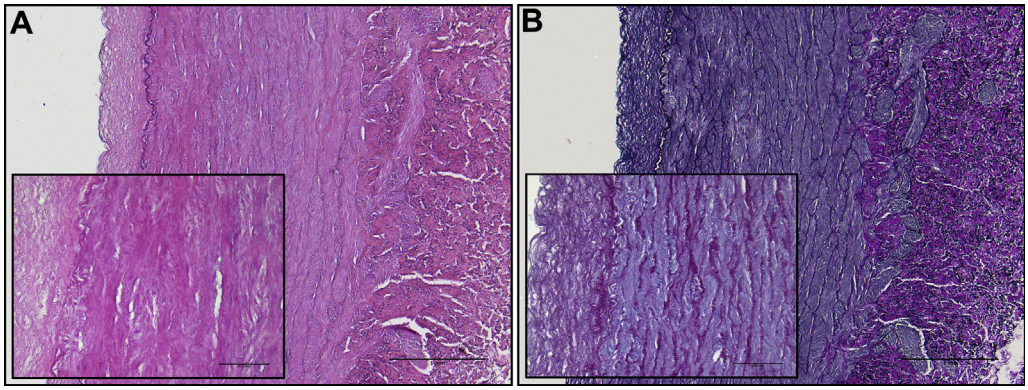

Fig. 2. Decellularized pig aorta before implantation. Hematoxylin and eosin (HE) **(A)** and Van Gieson's (VG) stain **(B)**; 50× − insert 200×.

Table II. Treatment group with survival

Group	Treatment	Number	Cause of death	Survivors (*n*)
A	Elastase-collagenase	4	Aortic thrombosis (1)	3
B	Elastase-collagenase-stenosis	7	Aortic thrombosis (3), pneumonia (1)	3
C	Xenograft	3	-	3
D	Xenograft-elastase-collagenase	4	Bleeding (3)	1

blood supply to the spinal cord. Finally, 18 sheep were operated on, 8 of them died shortly after the operation (Table II). Four were euthanized because of paraplegia due to aortic thrombosis shortly after protease infusion, 3 animals of group D died because of an aortic rupture within 24 hr after operation, and 1 animal died of a postoperative pneumonia.

Computed Tomography

Figure 3 depicts the CT measurements of each individual sheep. It shows that the variation between and within groups was large. In the elastase-collagenase stenosis group (B) and the xenograft group (C), there was a diameter increase after 6 weeks till the end of the experiment. A maximum dilatation of up to 30% was seen 24 weeks postoperatively in the protease with stenosis group. The diameter of the protease group (A) and xenograft-protease group (D) after 6 weeks was smaller than the starting diameter. In the subsequent weeks, the diameter of group A increased, but for the 1 animal in group D, the diameter remained smaller till the end of the experiment.

Microscopic Evaluation

The normal nonoperated control infrarenal aorta of a healthy sheep consists of a very thin intima, media, and adventitia. This is surrounded by periadventitial tissues consisting of loose connective tissue with fatty tissue (Fig. 4). Sampling directly after the operation in group A showed disappearance of the intima, influx of inflammatory cells, and mural thrombosis (Fig. 5). In the subsequent samples, there was a limited amount of inflammation, mild fragmentation of elastin fibers, formation of a neointima, and spots of calcification in the media.

Evaluation of samples of group B animals, which died within 24 hr after the operation, showed a luminal thrombus and edema and infiltration with inflammatory cells at the inner part of the media (Fig. 6). During follow-up (week 12 and 24), results were comparable to group A. Calcifications of media with foreign-body giant cell reaction, mild fragmentation of elastin fibers, and formation of a neointima were visible.

The first evaluation of group C, after 6 weeks, confirmed the decellularized scaffold with formation of a neointima and pronounced inflammatory and fibrotic reaction outside the vessel wall. Twelve weeks after implantation, an increase in foreign-body giant cell reaction at the outer border and influx of inflammatory cells and fibroblasts in the media with slight destruction of the scaffold was visible. Another 12 weeks later, a decrease in inflammatory reaction, full repopulation of the

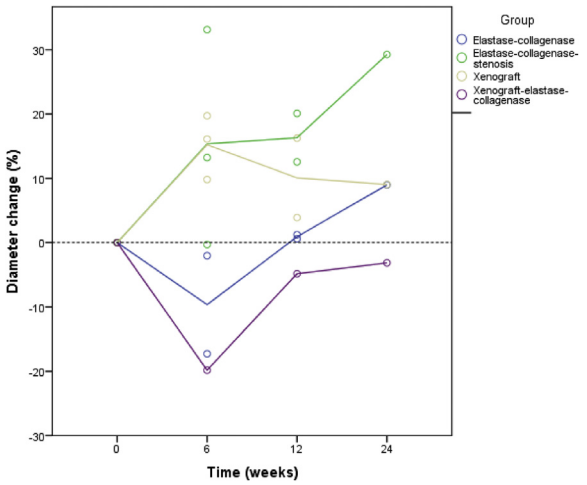

Fig. 3. Diameter change in time of the treated aorta. Each data point is the maximum diameter in anteroposterior direction of the infrarenal aorta of an individual sheep. Each line connects the average diameter per time point of a specific group.

media with fibroblasts, and thickened fibrotic adventitia with mild calcification was visualized (Fig. 7).

Evaluation of the samples of group D, which died because of bleeding shortly after operation, showed disappearance of the intima and luminal thrombus, a decellularized media, and edema and fragmentation of elastin fibers at the inner part of the media (Fig. 8). The samples 24 weeks after implantation showed the formation of a neointima, a thin media with deposition of collagen, and thickened adventitia with extensive fibrosis and no inflammation (Fig. 9).

DISCUSSION

This study tried to extrapolate rodent aneurysm models—protease infusion and xenograft implantation—to an ovine model. This is because of the beneficial characteristics of sheep for cardiovascular research. During preliminary experiments in 2 sheep, paraplegia occurred after the procedure without signs of aortic thrombosis. This dramatic event was solved using a passive aortic bypass during aortic clamping. The first model to be extrapolated was the protease infusion model. Boudghène et al.[16] were able to induce aortic aneurysms in dogs using elastase, although other research groups, such as Strindberg et al.[17], failed to produce an acceptable aneurysmal dilatation in a canine model. In the subsequent years, several modifications were proposed to optimize the diameter increase in pig models (Table I). The overall histological characteristics of these models were an intimal hyperplastic reaction, degradation of the elastic fibers, alterations in smooth muscle cells, and inflammatory infiltration. Some describe the occurrence of calcium depositions. The average aortic diameter increase in these studies was around 50 till 100%. One of the main issues is that in some of these studies the growth of the animals was not considered,[19,22,23] although this is important as these animals grow and gain weight very fast (0.75−1.0 kg/day). Miniature swine might be an alternative, but Marinov et al.[18] showed that elastase infusion in the infrarenal aorta destroyed the elastic network of the media, but no aneurysmal dilatation could be produced. To the best of our knowledge, no study was performed on miniature pigs using elastase-optimized protocols such as combination with collagenase, hyperinflation, or proximal aortic stenosis. The histological features of aforementioned studies were comparable to our results. In groups A and B, there was intima damage and thrombus formation early after the initial operation. In the subsequent weeks, there was a formation of neointima, cellular infiltration, mild fragmentation of elastin fibers, and calcium deposition. Unfortunately, no valid diameter increase was observed. The maximum diameter increase was 30% after 24 weeks in the protease-stenosis group. The used doses of elastase and collagenase were based on the study by Hynecek et al.[19] Comparing doses of proteases between studies is difficult, because frequently, brands and batches, and therefore the hydrolyzing effect per unit, are not specified. Moreover, evidence suggests that in a protease model not only the elastin destruction but also the presence of contaminators, which influence the inflammatory response, seem to play an important role in the immune-mediated reaction.[26] In the described large animal studies, long-term data, more than 6 weeks, are lacking. In this study, there was histological evidence that 5 months after the elastase-collagenase infusion, there is disappearance of the thrombus, formation of a neointima, decrease in inflammation, and increased fibrosis in the adventitia. This is in accordance with a follow-up study in elastase-induced aneurysm in rabbits showing signs of regeneration.[27] It seems that a certain time after the ''initiating'' event, regeneration stabilized the aortic tissue and prevented further diameter increase.

Allaire et al.[13] first described the use of xenografts. There is little literature regarding pig-to-sheep xenotransplants, although a vigorous immune response has been described after

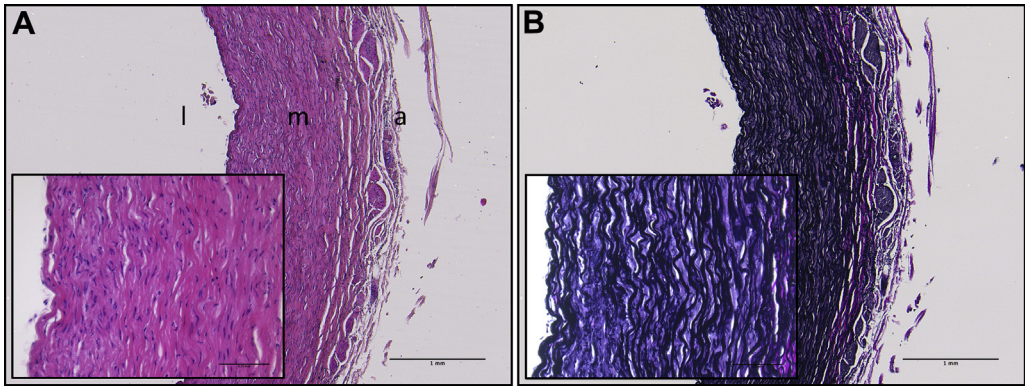

Fig. 4. Normal sheep aorta. HE **(A)** and VG stain **(B)**; 50× — insert 200×. l, lumen; m, media; a, adventitia.

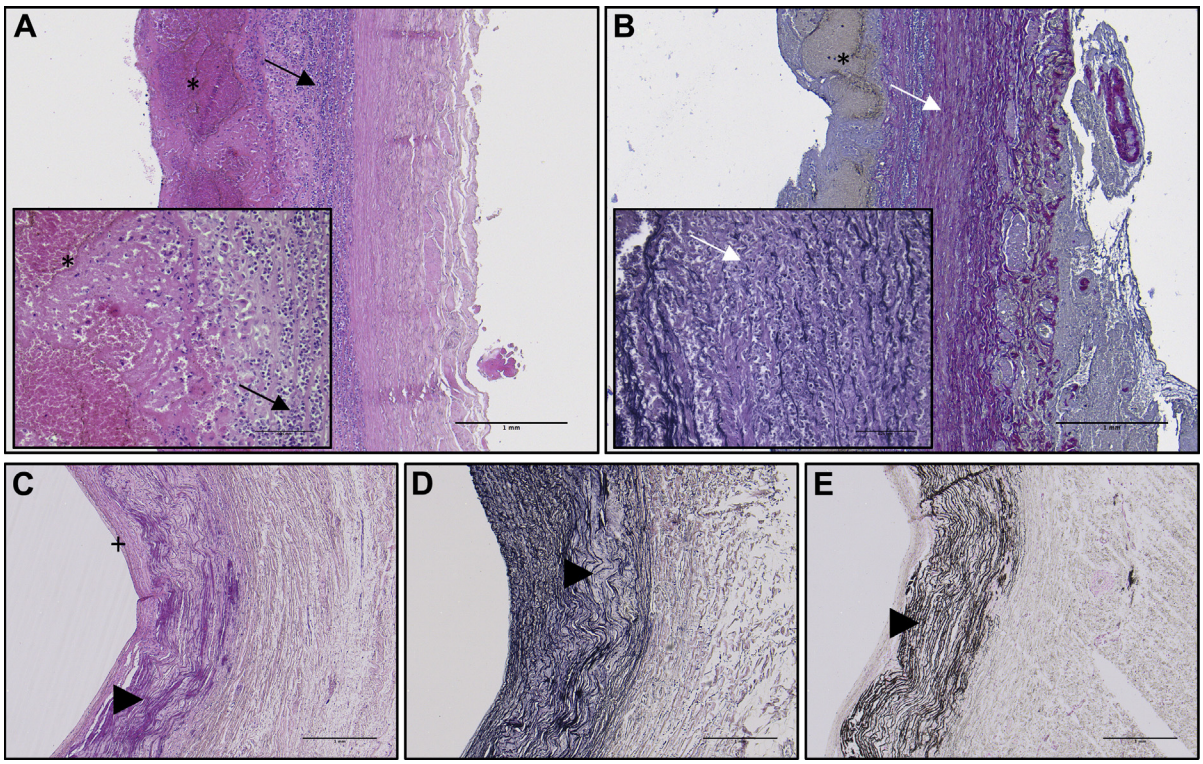

Fig. 5. Histological evaluation 1-day HE **(A)** and VG stain **(B)**; 50× — insert 200×; and 12 weeks HE **(C)**, VG stain **(D)**, and Von Kossa staining **(E)**; 50× after elastase-collagenase perfusion, group A. *, Thrombus; *black arrow*, inflammatory cells; *white arrow*, disruption elastin fibers; +, neointima; *arrowhead*, calcification.

pig-to-sheep heart transplant.[28] Six weeks after implantation, a pronounced inflammatory reaction from the adventitia was visible. In rodent xenograft models, histological changes are comparable.[14,29] By the end of the experiment, the degree of inflammation diminished, the media was repopulated with fibroblast, and the adventitial tissue contained a thick layer of collagen. These findings suggest, also after xenotransplantation, stabilization and healing of the aortic tissue. CT evaluation in this group showed no increase in aortic diameter in comparison with the start of the experiment. Recently, a large animal xenograft study implanted decellularized sheep aorta into pigs.[24] There was an increase in aortic diameter of 81 ± 30% in the following 47 days. Owing to the extensive weight gain in these

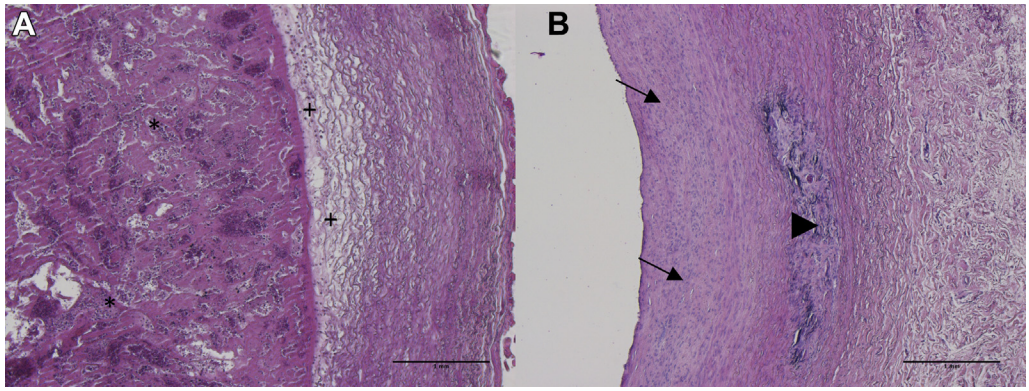

Fig. 6. HE staining 6 hr **(A)** and 12 weeks **(B)** after elastase-collagenase perfusion and creation of aortic stenosis, group B. *, Thrombus; +, edema; *arrow*, inflammatory cells; *arrowhead*, calcification (50×).

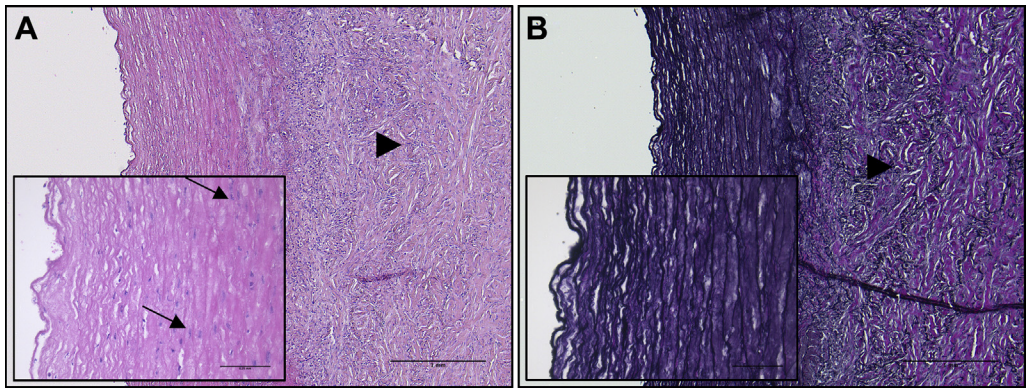

Fig. 7. Decellularized pig aorta, group C, 24 weeks after implantation, there is a repopulation of the media and thickened fibrotic adventitia. *Arrow*, fibroblasts; *arrowhead*, adventitia (50×; HE) HE **(A)** and VG stain **(B)**; 50× − insert 200×.

conventional pigs, longer follow-up was impossible. In contrast to our long-term results, no repopulation of the xenograft or signs of stabilization of the diameter were visible. The large diameter increase obtained in this study might have had diverse causes. First of all, the immune responses will be different because of the differences in species and age—young pigs versus full-grown sheep.[14,29] Secondly, bias might have been introduced by using only ultrasonographic measurements by different observers. Moreover, the reference diameter was the diameter just before and not after the transplantation. Because of the disappointing aortic diameter increase in our study, it was decided to combine the 2 procedures and treat the xenografts with an elastase-collagenase solution before implantation. Only one of the 4 animals survived the perioperative period. Three sheep died because of sudden hemodynamic collapse in the first hours after operation. At autopsy, large defects in the wall were visible. Already, peroperatively, it was difficult to control diffuse bleeding due to a very fragile aortic wall. Microscopic evaluation of those early failures showed an edematous media with extensive fragmentation of the elastin fibers. It was considered unethical to continue with this group as perioperative mortality was too high. On the other hand, the sheep that survived its predetermined period of 24 weeks showed some histological features of AAA with thinning of the media, inflammatory cells, and formation of neointima. On the contrary, the increase in aortic diameter was limited.

Another frequently described murine model, chronic Ang II infusion[10], was not used. It is difficult to extrapolate this to large animals as an enormous amount of angiotensin, and money, would be necessary to run such an experiment. Moreover,

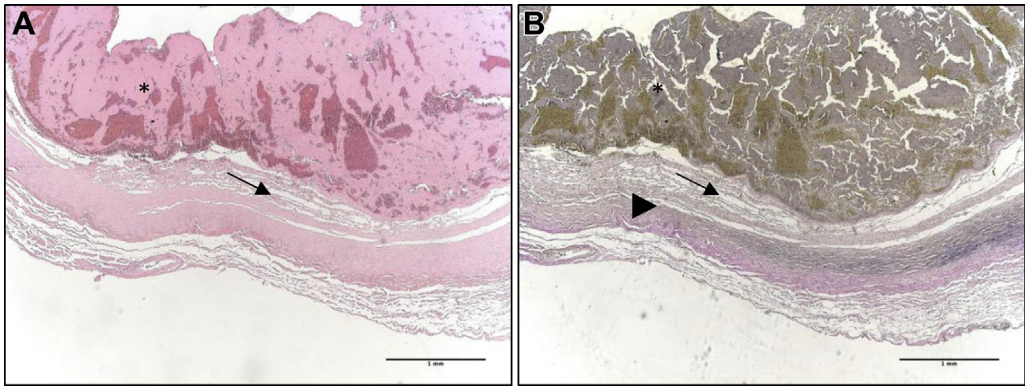

Fig. 8. Protease-treated xenograft within 24 hr after implantation. HE **(A)** and VG stain **(B)**. *, Thrombus; *black arrow*, edema; *arrowhead*, fragmentation elastin fibers (50×).

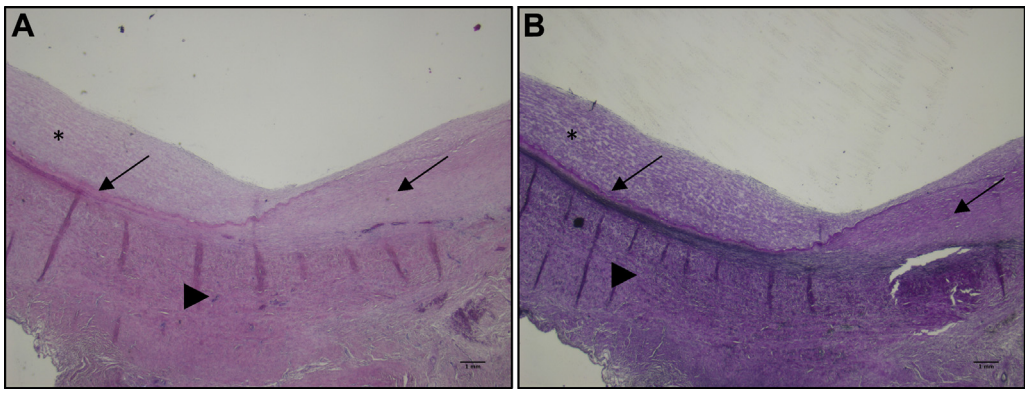

Fig. 9. Xenograft treated with elastase-collagenase, group D, 24 weeks after implantation. HE **(A)** and VG stain **(B)** shows neointima, local thinning of media, and thickened fibrotic adventitia. *, Intima; *arrow*, media; *arrowhead*, adventitia (12.5×).

recent research suggests that this is more of a dissection-based model.[30,31] Calcium chloride periaortic application was not used because intraluminal thrombus was never described in this model, a feature which is considered to be important in human AAA. Moreover, no study described a greater than 50% increase of the abdominal aorta in large animals using $CaCl_2$ alone.[12,32] To the contrary, it can even cause a decrease in relative aneurysm dimension over time.[22]

There are certain limitations in this study. The number of animals was low; moreover 3 random time points were chosen (6, 12, and 24 weeks). Owing to this small number, the histological evaluation was rather basic (1 animal per group per time point). For that reason was a quantitative evaluation deemed inappropriate, and a qualitative assessment by an experienced pathologist was performed. Another limitation is that only one dosing scheme of proteases was used. In the ideal scenario, more doses were evaluated. The CT-based aortic diameter within each group varied greatly. This could be because of intraobserver and interobserver variability caused by difficulties in defining the exact outer diameter, and not the luminal diameter, of the vessel. However, the overall changes in diameter were so low that it is very unlikely that a significant diameter increase was present. A sham-operated group was not included as the paired design using the initial diameter as a control, just before protease infusion or after transplantation potentially excludes other risks of confounding. Owing to the high perioperative mortality and the mild diameter increase, it was considered useless to increase the number of animals in one of the groups. Nevertheless, it is deemed important to share this study as it gives an overview of currently available large

animal aneurysm models and shares the experience of the quest for a more valid animal model. Such a model could help the development and evaluation of new diagnostic tools and treatments of AAAs.

To conclude, proteolytic degeneration of the aortic wall, inflammation and immune responses, wall stress, and molecular genetics are considered the most important mechanisms in the development of human AAA.[33] In this study, proteolytic degeneration and inflammation were initiated using an elastase-collagenase mixture, proven to be effective in small animal models; wall stress was induced using ligation, and an immune-mediated reaction was caused by xenotransplantation. None of these techniques or combinations proved to be effective to induce aneurysms from a diameter perspective in sheep. Some histological findings of human surgical specimens such as inflammation, thinning of the media, and loss of elastin could be reproduced. Although long-term follow-up indicated stabilization, and even regression, of the inflammatory process.

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