Long-gap esophageal atresia - The development of an experimental model of esophageal regeneration in vivo as an attempt to improve clinical outcome

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To Lisa, Klara and Kornelia

Alegatus sum, ergo sum

Abstract

Background: A subset (~8-15%) of the patients born with esophageal atresia (EA) suffer from a lack of esophageal tissue, which makes a primary anastomosis difficult to achieve. This is most common in patients born without a distal fistula between the trachea and the distal esophageal segment.

Purpose: To investigate the clinical course and outcome in patients born with long-gap esophageal atresia (LGEA) in the western region of Sweden and to develop an experimental model of guided tissue regeneration in the intrathoracic esophagus.

Methods: A retrospective study of 16 consecutive patients born with LGEA between 1995 and 2010 was performed. The patients had been followed according to a structured program at one and seven years of age. The experimental studies had been performed in growing piglets, where 3 cm of the intrathoracic esophagus had been replaced with a silicone stented Biodesign® mesh. The piglets were provided with a gastrostomy through a small midline laparotomy. Factors influencing the clinical and histological outcome had been recorded. In Paper II, six piglets underwent surgery, in Paper III, ten and, in Paper IV, six.

Results: No mortality was seen in the patients with LGEA. The mean age at definitive surgery was 147 days. The patients were small for gestational age. Eleven of sixteen (70%) had a delayed primary anastomosis as a definitive procedure, three had a gastroplasty and two underwent a colonic interposition. After surgery, anastomotic leakage was seen in seven of 16 (45%) patients and stricture developed in 11 of 16 (70%). At follow-up, some catch-up in weight was seen at seven years of age, but no catch-up in stature was seen.

Spirometry performed at one and seven years of age showed obstruction or restriction in 9 of 14 (55%) measurements. The spirometry findings did not indicate any further need for surgery. Multiple breath washout was within the normal range in 11 of 15 (75%) measurements at one and seven years of age. Three of four (75%) of the patients with a pathological lung clearance index (LCI) at multiple breath washout required further surgery to prevent pulmonary damage due to aspiration.

All patients either underwent surgery or were receiving continuous medical treatment for gastroesophageal reflux, and 7 of 16 (45%) had gastrostomy at the end of the study period. All patients were able to drink orally, but two of 16 (13%) were unable to eat solid foods.

In the first experimental study (Paper II), six animals lived for one to 17 weeks after surgery. Four animals were alive for at least four weeks and in two of them (50%) the stent was lost prior to four weeks. Piglets that lived longer than four weeks had recurrent stricture and required dilation. Histology showed connective tissue and intense angiogenesis in three piglets. In two of them, living four and 17 weeks respectively after surgery, the bridging area contained islets of immature-looking cells in the submucosa. The remaining three piglets only had inflammatory cells and fibrosis in the bridging area.

In Paper III, the piglets lived for three to 10 days after surgery. Three of 10 (30%) animals were sacrificed prior to plan due to mediastinitis. The surgical method was developed in such a way that the bridging graft could be sewn without leakage. If there was

no significant leakage, the bridging graft was macroscopically surrounded by a tissue tube that connected the native esophageal edges. Histology showed connective tissue and inflammatory cells with intense angiogenesis in the bridging area. In addition, a thin layer of smooth muscle cells was seen around the bridging graft. In the piglets with significant leakage, there was an aggressive inflammatory pattern, with macrophages in the native muscle layers and islets of lymphocytes in the bridging area.
In Paper IV, all the piglets survived until sacrifice 20 days after surgery. In two of six (33%) piglets, there was stent loss prior to sacrifice. In the animals with a retained stent, the tissue tube between the native muscle edges was easily dissected macroscopically. If the stent was lost, the bridging area was narrow and attached to the surrounding tissues with firm adhesions. Histology in the piglets with a retained stent showed that the bridging area was organized in three layers with islets of smooth muscle cells organized in two layers in the wall. CD163-positive, M2 machrophages were seen close to the lumen. In those animals in which the stent was lost, the organization into three layers could not be seen. There were no M2 macrophages in the specimen, but calprotectin-positive, M1 macrophages could be seen throughout the wall of the bridging area.
Conclusion: Patients in our study born with LGEA required long hospitalization and suffered from symptoms related to gatroesophageal reflux during childhood. These individuals were small for gestational age. Some catch-up in weight was seen at seven years of age, but no catch-up in stature was seen. Multiple breath washout might be a valuable tool for the early detection of aspiration into the lungs in this patient group. The experimental model for replacing a part of the intrathoracic esophagus in growing piglets showed that a remodeling inflammatory pattern, accumulation of muscle cells and a structured overall organization in the wall of the bridging graft can be achieved under favorable conditions. Leakage in the anastomoses and stent loss prior to 20 days changed the inflammatory profile and gave rise to scar tissue formation and stricture. Future studies are needed in order to see whether these differences account for a regenerative healing process, with functional tissue reforming in the esophagus.

Keywords: long-gap esophageal atresia, pulmonary physiology, esophageal replacement, guided tissue regeneration, macrophage phenotype, extracellular matrix

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Contents

List of publications

The thesis is based on the following articles:

I. **Jönsson, L. Friberg, LG. Gatzinsky, V. Kötz, K. Sillén, U. Abrahamsson, K.**
Treatment and follow-up of patients with long-gap esophageal atresia -15 years' experience from the western region of Sweden.
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II. **Jonsson, L. Gatzinsky, V. Jennische, E. Johansson, C. Nannmark, U. Friberg, L. G.**
Piglet Model for Studying Esophageal Regrowth after Resection and Interposition of a Silicone Stented Small Intestinal Submucosa Tube.
Eur Surg Res, 2011. 46:169–179.

III. **Jonsson, L. Friberg, L. G. Gatzinsky, V. Jennische, E. Sandin, A. Abrahamsson, K.**
Early regenerative response in the intrathoracic porcine esophagus-the impact of the inflammation.
Artificial Organs, 2014. 38: 439-46.

IV. **Jonsson L, MD, Dellenmark Blom M, RN, Friberg L-G, MD, Gatzinsky V, MD, Holmqvist O, MD, Jennische E, MD, Prof, Sandin A, MD,Abrahamsson K MD, Assoc Prof.**
Macrophage phenotype is associated with the regenerative response in experimental replacement of the porcine esophagus.
Submitted for publication, October 2014, Artificial Organs

List of Abbreviations

EA	Esophageal atresia
LGEA	Long-gap esophageal atresia
PVC	Polyvinylchloride
LES	Lower esophageal sphincter
ECM	Extracellular matrix
SIS	Small intestinal submucosa
VU	Vertebral units, a vertebral body with a disc.
RI	Reflux index
MBW	Multiple breath washout
LCI	Lung clearance index
FRC	Functional residual capacity
FEV1	Forced expiratory volume within 1 second
FVC	Functional vital capacity
TLC	Total lung capacity
SGA	Small for gestational age
LLMI	Lipid-laden macrophage index

Introduction

Esophageal atresia (EA) is a congenital malformation in which the separation of the foregut bud into the esophagus and the trachea is affected. There are five main subtypes of the malformation, depending on the presence of a fistula between the esophagus and the trachea, where it is situated and whether or not there is a discontinuity in the esophagus (1). In about 8% of the patients there is no fistula from the lower esophageal segment and the trachea. These patents often have a lack of esophageal tissue, which makes an esophageal anastomosis difficult (2,3,4). Today, these patients are treated with a delayed primary anastomosis or by some kind of replacement (5,6). These procedures involve long hospitalization and increased surgical risks compared with when a primary anastomosis can be achieved (7).

Experimental esophageal replacement in animals has been developed so that patch repair can be performed with the regeneration of functional esophageal tissue , but strictures and scars always occur after circumferential resections of the thoracic esophagus (8,9,10,11).

Recent advances in regenerative medicine have shown that the inflammatory pattern in a healing wound can be adjusted so that functional tissue, rather than scarring, is formed during the healing process(12,13). Further studies of circumferential resections of the thoracic esophagus are needed to develop a treatment for replacing the esophagus in EA patients with a lack of tissue, in order to enhance the function and reduce the surgical risks.

Background

Esophageal atresia

History

Esophageal atresia (EA) was first described in 1670 by William Durston, but it was not until 1935 that the first child born with esophageal atresia survived (14,15). Since the 1940s, when the first successful primary anastomosis was performed, survival has increased to more than 90% (3,16). In recent years, the focus has shifted to the morbidity present among these patients and how to diminish it.

Etiology

EA is the result of a defective separation between the esophagus and the trachea when the foregut develops into these structures. It is known that there is a slightly increased risk in the siblings of affected individuals, suggesting that there is a genetic factor. However, the majority of cases are sporadic. (17,18)

Epidemiology

EA has a described incidence of between 3-4/10,000 newborns (3,19). It is slightly more common in males, among Caucasians and with increasing maternal age(20,21).

Classification

The most commonly used classification of the different types of esophageal atresia is the classification proposed by Gross in 1953(1).

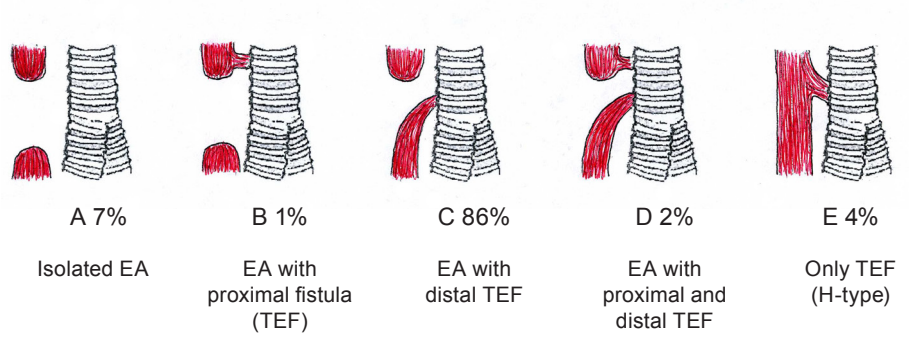

Figure 1. Classification according to Gross, with the proportion of each subtype. With permission Vladimir Gatzinsky

In order to describe the difficult cases without a distal fistula (Gross A+B), the term long-gap esophageal atresia (LGEA) is used (3,23,24). The definition of LGEA is controversial. At first, the definition was only used for patients born without a distal fistula (Gross A+B) and with a short distal pouch which prevented a primary anas-

tomosis (2,22,23). Later on, many authors used the term for all patients in whom a primary anastomosis could not be performed in the neonatal period (4,24,25).

Associated anomalies

Associated malformations are commonly seen in patients born with EA and affect as many as 50% of the patients. The incidence is higher in individuals without a distal fistula compared with the others. About 5% of patients have chromosomal abnormalities (26).

Diagnosis

EA is suspected when a newborn has excessive salivation and regurgitates, or chokes and coughs when fed. The inability to pass a feeding tube into the stomach raises the suspicion still further. A plain X-ray of the chest and abdomen often produces the diagnosis[Figure 2].

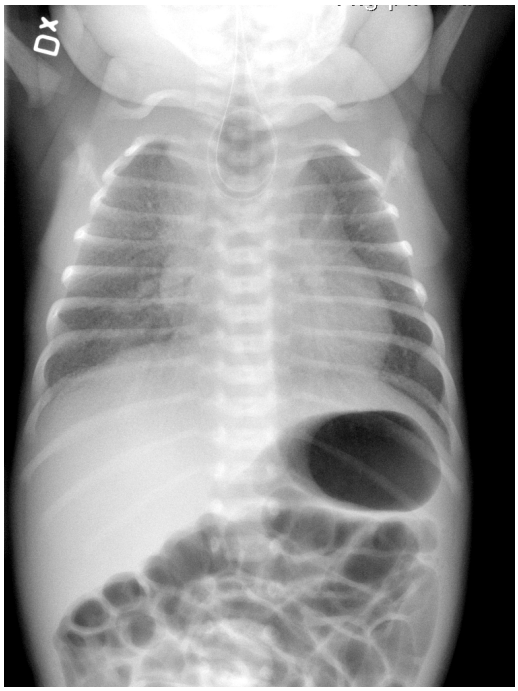

Figure 2. Plain x-ray of a patient born with esophageal atresia.

If the diagnosis is still unclear, an esophagogram with contrast installation confirms the diagnosis. If the abdomen is gasless, there is no connection between the lower esophageal segment and the airways.

Detection of the malformation antenatally is difficult, but it may be suspected if the ventricle is absent or small for age, combined with the presence of polyhydramniosis. If a blind-ended upper esophageal pouch can be visualized by ultrasound, this has been shown to have a positive predictive value of 100% (27).

Surgery

If the patient has gas in the abdomen, showing that there is a distal fistula, the first surgical procedure is a thoracotomy with the aim of achieving a primary anastomosis. If the abdomen is gasless, a laparotomy is performed with measurements of the gap, with something radiopaque in both the esophageal segments [Figure 3].

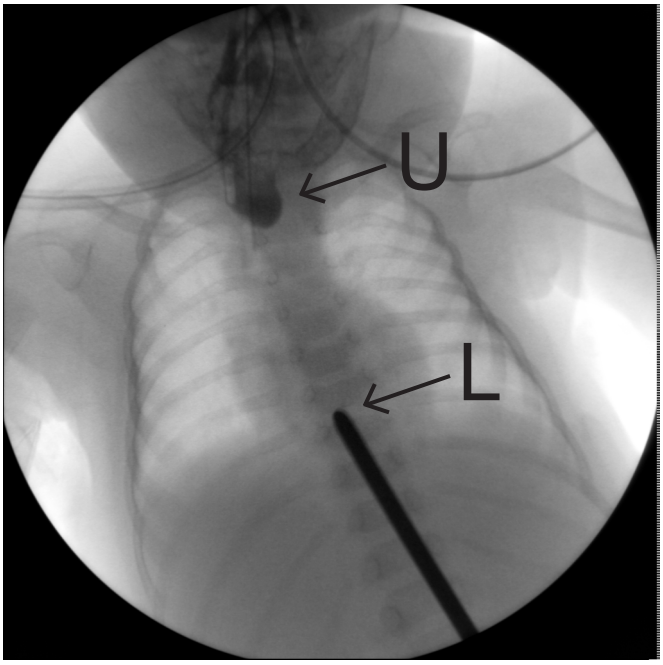

Figure 3. Fluoroscopic picture of a long gap esophageal atresia patient with a Hegar-dilator in the lower esophageal pouch(L) and contrast agent in the upper pouch(U).

Some authors advocate endoscopic cannulation of the distal segment, while others recommend a dynamometer in order to have fixed pressure when measuring (4,28). If the gap is less than 2 VUs, a primary anastomosis can be performed. Otherwise, a gastrostomy is constructed and a suction catheter is placed in the upper esophageal pouch, to drain the saliva. The patients are nursed on the ward and regular measurements of the gap are made. As the lower esophageal segment tends to grow more rapidly than the child in the first months of life, the gap often diminishes, which allows for a delayed primary anastomosis (3,6). If the gap remains too large to bridge, elongation procedures on the upper pouch (29,30), gastroplasty (31,32,33) or esophageal replacement can be performed. Some of the methods are only suitable for bridging small gaps, whereas others can replace the entire esophagus from the neck to the abdomen, i.e. an esophageal replacement. When it comes to esophageal replacements, the small bowel, colon and stomach can be used (5,34,35,36). When the stomach is used, either a total gastric pull-up or a gastric tube can be constructed from the major side of the ventricle (35,37). All these methods have increased mortality and morbidity compared with a patient in whom a primary anastomosis can

be performed (5). There is no consensus in the pediatric surgical community on which method should be used. Foker et al. have shown that it is possible to close the gap by traction of the esophageal pouches over a period of days (38). In a follow-up study, the patients had good function in the esophagus and good quality of life (39). The method has not worked this well at all institutions and the results appear to be in line with, or even worse than other methods for reconstructing LGEA (24,40).

If there is a distal fistula and a thoracotomy is performed, there is sometimes still a lack of tissue, which makes a primary anastomosis difficult (3,4). The same lengthening procedures as those described above can be used to achieve sufficient length for a primary anastomosis. Another strategy is to close the fistula between the distal esophageal pouch and the airways and then construct a gastrostomy. The patient can then be fed through the gastrostomy, while waiting for the final reconstruction. This approach is mainly advocated for very low birth weight patients, where more excessive surgery is deemed too challenging (41,42). Any further growth of the esophageal pouches has never been documented in this situation, but the child will grow larger and this will hopefully facilitate the definitive surgery.

Outcome

The overall survival rate nowadays is more than 90%, with most of the deaths attributed to non-avoidable causes. There are sporadic cases of death due to complications of the malformation and its treatment (7,43,44). Morbidity associated with the malformation and its treatment are common. Short-term morbidity is mainly associated with the surgical procedure and includes anastomotic leakage, stricture, recurrent fistula, thrombosis and sepsis (7,43,44,45,46,47).

Long-term morbidity originates from both the airways and the upper gastrointestinal tract. Dysphagia and gastroesophageal reflux are the most common problems from the gastrointestinal tract. Failure to thrive, or the need for sustained nutrition through a gastrostomy are more serious problems which can arise in more difficult cases. The risk of both short- and long-term morbidity from the gastrointestinal tract increases in patients in whom a primary anastomosis is difficult to achieve (7,44,45,48,49). When an esophageal replacement is performed, mortality rates of 0-15% have been described (5,50). In the few comparative studies performed between different methods, the complication rates vary and there is no consensus on which method to use (5,51,52). Traction elongation of the esophageal pouches prior to anastomosis appeared to be very good when performed by Dr Foker (39), but other groups have had difficulty reproducing his astonishing results (40,45,49). In an animal model, where esophageal traction was applied to the rat esophagus, the esophagus was longer and heavier in the rats exposed to traction of the distal part of the esophagus. Histology showed a preserved structure but also the presence of torn muscle fibers and scarring (53). Further follow-up time is needed before it can be shown that the elongated esophagus stimulated by traction is of value to LGEA patients.

Airway symptoms are also common after repaired EA (43,54). Animal studies have implied that the airways are included in the malformation, with pulmonary hypoplasia and tracheomalacia as inherent parts (55,56). In humans, these changes probably account for some of the airway symptoms. Dysphagia and gastroesophageal reflux are other causes (56,57,58). Tracheomalacia has been shown to be strongly associated with EA with a fistula (Gross B,C,D and E) (59,60). To our knowledge, as EA Gross C is by far the most common type of EA, all pulmonary function studies have been performed in this patient group (54,57,61,62).
The extent to which each of these factors contributes to the airway problems in each subgroup of EA patients is therefore still unclear.

Experimental models for esophageal replacement

As mortality and morbidity have been high after esophageal resections, whatever the cause, interest in developing better replacements has been shown since the mid-20th century. The first successful replacement of 5 cm of the intrathoracic esophagus in an experimental model was performed by Berman in 1952 (63). Using a PVC tube, he was able to bridge a 5 cm gap in the lower part of the esophagus. The animal survived and developed a tube made of scar tissue around the bridging graft. Berman treated 60 patients undergoing esophageal resections due to cancer with this bridging graft and reported low rates of acute complications, even if the long-term mortality from the disease remained high (64).

In 1962, Boyd used different artificial prothesis to bridge a 5 cm gap in the esophagus of pigs(65). He concluded that pigs were better animals for studying esophageal replacements, as their muscle composition in the esophageal wall is similar to that of humans, with skeletal muscle in the most proximal part of the esophagus, smooth muscle in the distal part and a mixture of both in between (66,67,68). Stricture remained a significant problem in his model(65). Both dogs and rodents have skeletal muscle all the way down to the lower esophageal LES, which might affect the way the tissue heals after damage, thereby making them less suitable for developing treatments designed for use in humans.
In 1965, Lister et al. used a bridging graft composed of a Marlesh® mesh and part of the rectus fascia to bridge a 3 cm long gap in the esophagus of growing puppies. If the stent was lost prior to 3 months, stricture occurred. However, if the graft stayed in place for that time, the animals developed well. At sacrifice, the bridging area was composed of scar tissue, but the length of the scar diminished with time. After nine months, only a narrow scar remained between the distal and proximal muscle edges (69).

Yamamoto et al. developed a canine model in the 1990s in which they used a silicone tube with precipitated collagen around it as a bridging graft. With this graft, they were able to bridge 5 cm long defects on the neck with perfect histological end results, whereas a scar always developed when the replacement was performed in the thorax (11,70). When they seeded the graft with cells from the buccal mucosa, they

obtained faster coverage with mucosa but not better histology in the external muscle coat. Wrapping omentum around the bridging graft gave rise to more complications and much poorer healing in the area (71,72).
At the end of the 20th century, sheets of extracellular matrix (ECM) extracted from different tissues in animals/humans were studied as replacements in different organ systems (73,74,75). Used as a replacement in dogs, rats and pigs, almost total restitution of the esophageal wall was achieved after patch repair. However, stricture was inevitable after circumferential resections (8,9,10). One of the first commercially available ECMs was extracted from small intestinal submucosa (SIS). SIS is available as sheets, which were initially called Surgisis®. Due to some structural enhancements (more fat and DNA extracted from the ECM), the product name was changed to Biodesign® (76).

When ECM meshes have been studied in different replacement models, attention has been drawn to differences in the immune response to the replacement and particularly to differences in the macrophage phenotype (12,13). When the macrophages are classically activated, i.e. M1 macrophages, they promote further inflammation and the healing process ends up with scarring. When the macrophages are activated by other microbiological signals, they develop a different phenotype and are called M2 macrophages (12,13,77). These different activation modes are not two discrete phenotypes but rather a continuum of different activation states. Never the less, the division into M1 and M2 phenotypes has been able to explain differences in both histological and clinical outcome in studies of both disease and wound healing (74,78,79,80,81). When ECM-derived meshes have been used, the presence of M2 macrophages has been favorable when it comes to achieving "reconstructive remodeling", i.e. the reformation of functional tissue, rather than just scarring (74,78,80).

Aims

To describe the outcome for LGEA patients and to develop an experimental model of esophageal replacement aimed at improving the outcome after surgery in patients with LGEA

The specific aims in each paper were as follows.

Paper I:
To describe the clinical features, surgical treatment and outcome after one and seven years in patients treated for LGEA

Paper II:
To develop an experimental model to study the replacement of a part of the intrathoracic esophagus in growing piglets and to describe the clinical and histological outcome

Paper III:
To evaluate factors influencing the histological and clinical outcome 10 days after surgery and to develop the surgical technique to achieve optimal outcome

Paper IV:
To evaluate factors which affect the histological and clinical outcome 20 days after surgery, with special emphasis on the inflammatory pattern

Material and method

Clinical study (Paper I)

All patients, eight boys and eight girls, born with LGEA between 1995 and 2010 in the western region of Sweden (2.5 million inhabitants), were consecutively included in this retrospective observational study. Ten of 16 (60%) had associated malformations and 3/16 (20%) were twins.

LGEA was defined as an abdominal X-ray with a gasless abdomen, combined with a distance between the upper and lower esophageal segment that was ≥ 4 vertebral units (VU) at the first measurement.

The median follow-up period was 6.5 years (range 2-15). The patients had been followed according to a structured follow-up program, where physiological tests had been performed at one and seven years of age. Weight and stature had been recorded regularly when the patients had been in contact with the hospital.

Gastroesophageal reflux disease was defined as poor function in the lower esophageal sphincter, causing symptoms such as retrosternal pain (heartburn), regurgitation, vomiting, poor weight gain and/or recurrent airway infections. The monitoring of 24-hour pH was performed using the Synmed system. From the 24-hour pH monitoring, a reflux index (RI) was calculated, as the time with a pH lower than 4 divided by the total time of the investigation in the distal part of the esophagus. An RI above 10% was considered pathological in the patients one year of age and an RI above 4% was considered pathological in the seven-year-old patients (82).

Weight and height development over time is compared with the normal population and also with other patients born small for gestational age.

Lung function was assessed with spirometry and multiple breath washout (MBW) at one and seven years of age (83,84,85,86). From the spirometry, an obstructive or restrictive breathing pattern was assessed and, from the multiple breath washout, a lung clearance index (LCI) was calculated.

At one year of age, the tidal breathing time to peak flow related to total expiration time, the maximum flow at FRC during tidal-volume rapid thoracic compression and the shape of the tidal breathing flow-volume curve were assessed. At seven years of age, the FEV1/FVC ratio, mean expiratory flow (ml/s) and reversibility (increase in FEV1 after an inhalation of salbutamol (%)) were used to assess the airway obstruction. If two of three of these had changes indicating an obstruction in the airways, the patients were deemed to have an obstructive ventilatory disorder.

A restrictive ventilatory disorder was suspected at one year of age if the FRC was > -2SD. At seven years of age, a restrictive ventilatory disorder was defined as a TLC of < -2SD or the combination of FEV1 and FVC < -2SD, together with a normal or high FEV1/FVC ratio. The presence of normal TLC ruled out a restrictive disorder.

MBW is a method for assessing pulmonary ventilation inhomogeneity as a function of the peripheral airways. To summarize, an inert marker gas (sulfur hexafluo-

ride) was inhaled during tidal breathing via a mouthpiece until an equilibrated gas concentration of 4% in inspiration and expiration was reached. In infants, a face mask was used and the patients were sedated with chloral hydrate (70 mg/kg body weight). After removing the inert gas, the gas was washed out from the lungs by the tidal breathing of room air. The cumulative expired volume necessary to reduce the end-tidal inert gas concentration from 4% to 0.1% was divided by the functional respiratory capacity (FRC). This ratio is expressed as the lung clearance index (LCI). The mean value of three recordings is reported and given in standard deviations related to results from healthy children(87). Gas concentrations were measured using a mass spectrometer.

Bronchoscopy and bronchoalveolar lavage were performed in patients with significant symptoms from the lungs or esophagus, prior to surgical intervention. These investigations were performed under general anesthesia.

The data and results for the growth of the patients were calculated in SD scores for the study group against the norm populations (general, twins, Mb Down and SGA populations). Comparisons with norm populations were performed using Wilcoxon's signed-rank test applied to the SD scores. Changes in SD scores over time were analyzed using Wilcoxon's signed-rank test. All the tests were two-tailed and conducted at the 5% significance level. The data were analyzed using version 9 of the SAS System for Windows.

Experimental studies (Papers II, III and IV)

In the experimental work, a model for the esophageal replacement of the thoracic esophagus through a right-sided thoracotomy had been developed. The resection was performed at the level of the tracheal branching into main bronchi, as this is the level at which esophageal tissue is missing in LGEA patients. The bridging graft, composed of a 4-ply Biodesign® mesh, sewn around a silicone tube, had been anchored using telescopic techniques at both the proximal and the distal end to avoid leakage [Figure 4].

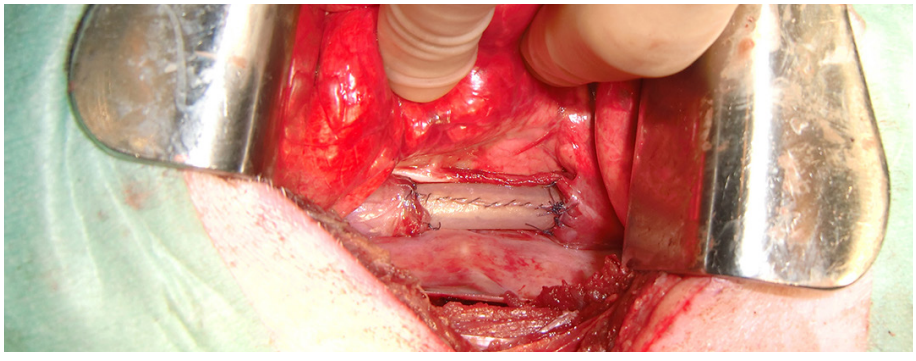

Figure 4. Bridging graft sewn into the esophagus in an experimental animal. Gap length between the muscles edges are 3 cm.

The piglets were put in the supine position and a small laparotomy was performed to construct a gastrostomy using an 18 to 26 ch Nelaton catheter, tunneled subcutaneously to the back of the pig [Figure 5].

Figure 5. Experimental animal in the animal housing a week after surgery. The gastrostomy catheter used for feeding is seen under the yellow wrapping.

Piperacillin 200 mg/kg + Tazobactam were given preoperatively and on the first postoperative day. A total of three doses were given. The piglets were fed through the gastrostomy until oral feeding was introduced. Rimadyl® (Carprofen) and Temgesic® (Buprenorphine) were given postoperatively to avoid postoperative pain. The animals had no bedding, to avoid the accumulation of bedding material in the esophagus, giving rise to obstruction. Two animals shared a room, but they were divided by a fence as long as they had a gastrostomy, to avoid damage. They were euthanized according to schedule after surgery, or if they showed signs of complications, such as leakage and stricture. After sacrifice, an autopsy was performed in which the bridging area was evaluated macroscopically and fixated for immunohistochemical analysis, as well as for electron microscopy.

In Paper II, six piglets underwent surgery. The bridging graft was constructed by suturing a 4-ply Biodesign® mesh around a 1.5 mm thick, 8 cm long silicone tube, with an outer diameter of one cm. Both the proximal and distal anastomoses were sewn with the mucosa and the external muscle coat at the same position, 1.5 cm from the center of the graft, using eight interrupted sutures of 5-0 monocryl (Ethicon®) [Figure 6a]. The silicone tube was anchored with two sutures of 3-0 Ethilon® at the proximal end and two sutures of 3-0 Vicryl® at the distal anastomosis.

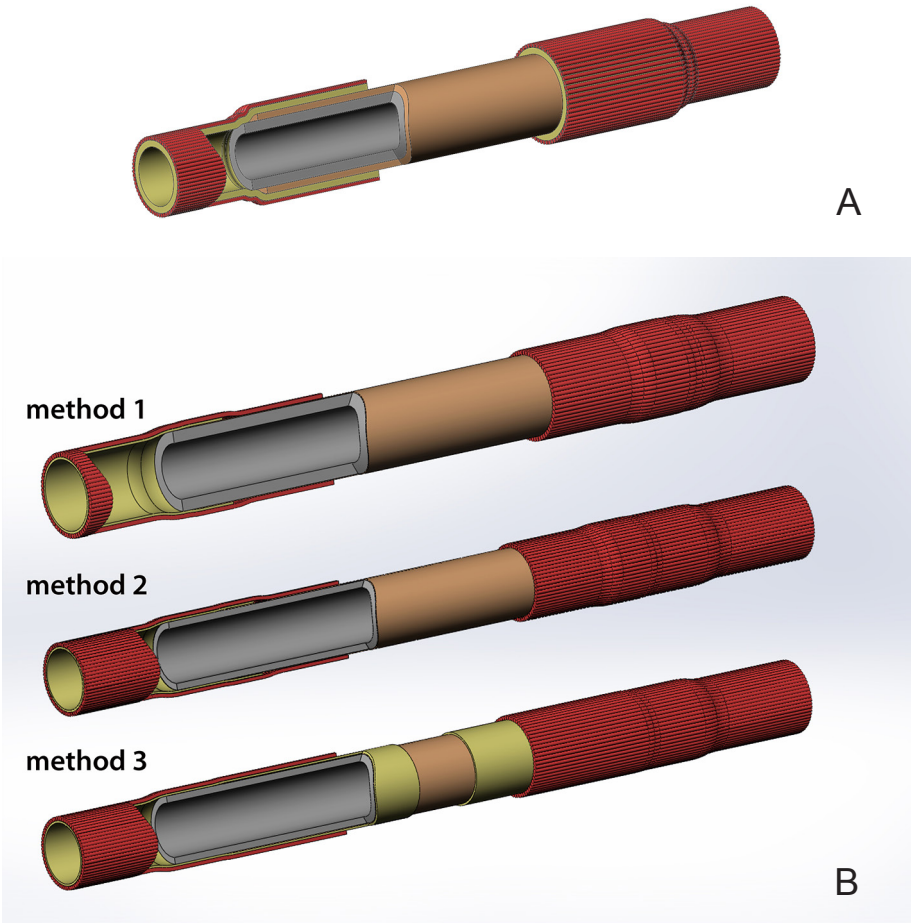

Figure 6. Schematic drawings of the fixation of the bridging graft in the experimental model. The mucosa is yellow, the esophageal muscle coat red, the Biodesign mesh is beige and the silicone stent is gray in the drawing. A) Fixation performed in paper II. The mucosa and the external muscle coat was sewn with interrupted sutures in one layer B) Fixation performed in paper 3. The mucosa was sewn with interrupted sutures in method 1, and with running sutures in method 2 and 3. Method 2 was used for the animals in paper 4.

The piglets were fed through the gastrostomy for four weeks. Dilation of the esophagus was carried out when a pig showed signs of stricture: increased salivation, longer feeding time and vomiting. Esophageal videofluoroscopy was performed using a Philips fluoroscope, with the animal in a wooden cage [Figure 7a,b]. The piglets were sacrificed at 1, 3, 4, 6, 8 and 17 weeks respectively.

In Paper III, 10 piglets underwent surgery. The first four piglets had a 60 Shore-A silicone stent, 7.5 cm long, 10 mm wide, with a 1.5 mm thick wall. Shore-A defines

Figure 7. Set-up for fluoroscopic examinations of the esophagus in the experimental study II.

the hardness of the silicone stent. When applied, the mucosa was sewn 5 mm from the edges and the musculature about 2 cm from the edges, in order to have a large zone of contact between the esophageal musculature and the Biodesign® mesh. The anastomosis was sewn with four to five interrupted sutures of 4-0 Biosyn at both ends. The stent was fixated both distally and proximally with Neurolon 2-0 [Method 1,Figure 6b]. The second four piglets had a bridging graft made of a 4-ply Biodesign mesh, sewn around a 60 Shore-A, 9 cm long, 10 mm wide silicone stent, with a 1 mm thick wall. The anastomosis between the esophageal mucosa and the Biodesign mesh was sewn with running sutures with biosyn 4-0 0.5cm from the edges of the

bridging graft. The musculature was fixed 2 cm from the end of the Biodesign mesh with interrupted sutures of Biosyn 4-0. The stent was fixed with one suture of Neurolon 2-0 both distally and proximally (Method 2,Figure 6B).
The last two piglets had a bridging graft made of a 4-ply Biodesign mesh, sewn around a 60 Shore-A, 9 cm long, 10 mm wide silicone stent, with a 1 mm thick wall. The esophagus was divided and a total of 3 cm of the musculature was resected after the mucosa had been retracted into the lumen. In this way, we made sure that more muscle than mucosa was resected. The mucosa was attached to the Biodesign mesh 0,5 cm away from the center with running sutures of Biosyn 4-0. The musculature was fixed together with the silicone stent with Neurolon 2-0 in order to make sure that no musculature was in contact with the Biodesign mesh (Method 3,Figure 6B). The animals were fed though gastrostomy until sacrifice. Sacrifice was planned 7-10 days after surgery for practical purposes. If there was signs of mediastinitis, the animals were euthanized prior to the planned date. Histology was performed using immunohistochemistry to map out the distribution of smooth-muscle cells, inflammatory cells and the mucosa in the bridging area.

In Paper IV, six piglets underwent surgery according to Method 2 in Paper III (Method 2,Figure 6B) and they lived for 20 days after surgery. They were fed through gastrostomy for 10 days and ate probe formula orally for another 10 days prior to sacrifice. Nexium® was given orally to piglets 3-6 in order to avoid acid-related damage to the healing area and to relieve acid-associated symptoms in the animals.
In animals 5 and 6, pledgets were used to secure the silicone stent to avoid stent loss prior to 20 days. Immunohistochemistry was performed to map the distribution of connective tissue, smooth-muscle cells, inflammatory cells and nerves. Selective antibodies were used to distinguish proinflammatory M1 macrophages from remodeling M2 macrophages.

All the studies were conducted according to good clinical standards and with ethical permission from the animal ethics committee of the western region of Sweden.

Table 2. Shows the antibodies used to visualize the different cell types and extracellular components.

Cells/ extracellular component stained	Histological marker
Pericyte, Smooth mucle cell, Skeletal muscle cell	Desmin
Smooth muscle cells, myofibroblasts	Smooth muscle actin
Smooth muscle cells	Smooth muscle myocin
Basal membrane	Laminin
T-Lymphocytes	CD3
M1-macrophages, neutrophil granulocytes	Calprotectin
M2-macrophage	CD163
Small synaptic vesicles, nerve specific	Synaptophycin

Results

Clinical study (Paper I)

Surgery

The mean age at definitive surgery was 147 days(Range 60-240). Eleven patients, in whom the segments had grown to a distance of less than two VUs, had a delayed primary anastomosis. Three cases had a gastroplasty and two a colonic interposition. We have used gastroplasty when the patients only needed a shorter elongation in order to achieve an anastomosis and a colonic interposition when a longer defect had to be bridged. Five of 16 (30%) patients required further surgical procedures due to gastroesophageal reflux.

Eleven of 16 (79%) patients required three or more dilations during the study period and anastomotic leakage was seen in 7/16 (45%) patients. In the patients undergoing delayed primary anastomosis, 6/11 (55%) had an anastomotic leakage, where two required a re-operation with resutering and open drain placement.
At the end of the study period, 14/16 patients were on continuous PPI treatment, while the other two were among the five patients who had reflux surgery performed after the definitive repair.

pH monitoring at one and seven years of age did not correspond to the future need for surgical intervention. At the end of the study period, 7/16 still had a gastrostomy to aid nutrition. All the patients were able to drink orally, but two were unable to eat solid foods.

Growth

At birth, the patients were small in weight, -2.3 SD (Range -4 - 0,2) and stature, -1.1 SD (Range -4 - 1.2) for gestational age and they continued to be small at one (Weight -2 SD(Range -3.2 - 0), Stature -1.7 SD(Range -4.5 - 0.8)) and seven (Weight -1.1 SD(Range -3 - 0), Stature -1.7 SD(Range -3.2 - 0) years of age. There was a statistically significant catch-up in weight between birth and seven years of age, but no catch-up in stature. [Figure 8 A,B]

Lung function

Pulmonary physiology was performed in seven patients at one year of age and in eight patients at seven years of age. One patient at the beginning of the series declined testing, while one patient at the end of the series was not tested in the study period due to severe comorbidity. One patient was tested at both one and seven years of age. The LCI was normal in 11 measurements and increased in four. Three of the four patients with an elevated LCI required further surgery to avoid gastroesophageal reflux and/or aspiration. At the age of one year, four of seven patients (57%) had signs of airway obstruction in spirometry, but only one of these needed treatment. At the age of seven years, three of eight patients (38%) had airway obstruction at

spirometry. At one year of age, no patient displayed a restricted ventilation pattern, but this was noted in two patients at seven years of age.
Bronchoscopy had been performed as part of the gastroesophageal reflux evaluation in 6/16 patients. In three of six patients (nos 7, 10, 11), the lipid-laden macrophage index (LLMI) was ≤ 20. Intensified treatment with inhalations relieved their symptoms and no further anti-reflux surgery was performed to protect their airways. In two of six patients (nos. 3, 14), the LLMI was 48 and 50 respectively. Aspiration was suspected due to the clinical picture and other investigations. Further surgery was required to avoid progressive pulmonary damage. In one patient (no. 6), food was seen in the airways and surgery was performed to prevent further aspiration.

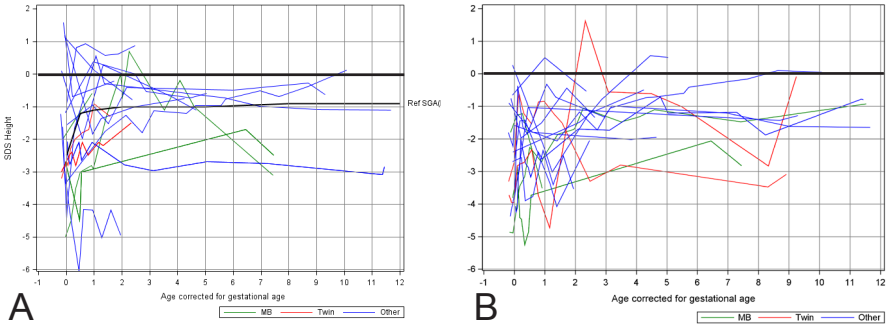

Figure 8. Height and weight development in LGEA patients given in SDS. The height and weight have been normalized for gender, Mb Down and twins. The Ref SGA(L) line seen in A is computed from data relating to the growth of children born short for gestational age. A) Height SDS B) Weight SDS.

Experimental studies (Papers II, III and IV)

In Paper II, all the pigs survived surgery and gained weight proportionally to their age during the study period. Four of six animals lived for longer than four weeks and, in this group, stent loss was 2/4. There were no cases of leakage from the esophageal anastomosis that required intervention, even though all the piglets showed signs of mediastinitis during the first week after surgery, with fever, gastrointestinal paralysis and vomiting.
After the stent was lost, stricture developed in all animals and it was treated with dilations in three animals [Figure 9]. In piglets nos 1, 3 and 6, histology showed fibrous tissue in the bridging area but also islets of desmin-positive cells, with a large nucleus and sparse amounts of organelles at electron microscopy [Figure 10,11,12]. Their appearance in electron microscopy was similar to pericytes seen in sprouting vessels (88), but here the cells appeared in clusters in the esophageal wall, not in the vessel wall. In piglet no 6. elongated desmin-positive cells, resembling mature smooth-muscle cells, could be seen in the bridging area [Figure 13]. In piglets nos 2, 4 and 5, only connective tissue and inflammatory cells were seen.

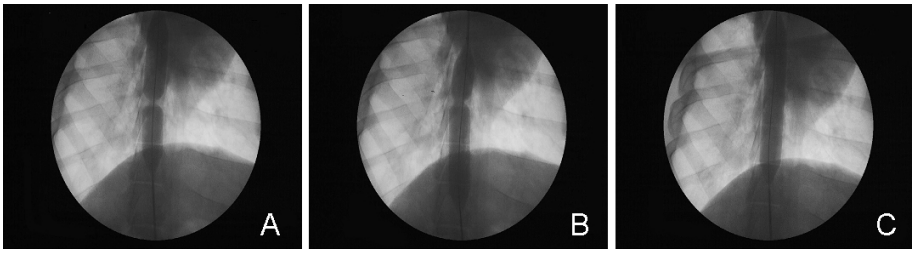

Figure 9. Fluoroscopic images of a dilation in an experimental animal in Paper II. A Bard® balloon catheter has been used, and a pressure of 2,5 atmospheres. A) Before dilation. B) In the middle of the dilation c) After Dilation.

In Paper III, 10 piglets underwent surgery. Three of four piglets undergoing surgery according to Method 1 had leakage, which gave rise to sacrifice due to signs of mediastinitis or fistulation between the esophagus and the thoracotomy wound. Adjustment of the suturing technique and silicone tube was performed for the next four piglets (Method 2). They had no clinical signs of leakage and, at sacrifice, a macroscopic tissue tube bridged the gap between the proximal and distal esophageal edges [Figure 14]. Piglets nos 9 and 10 underwent surgery according to Method 3, both had significant leakage and no 9 was sacrificed prior to plan due to symptoms of mediastinitis. Histology showed that the bridging area consisted of a layer of inflammatory cells towards the esophageal lumen. Towards the adventitia, there was a layer consisting of inflammatory cells, sprouting vessels and smooth-muscle cells. Longitudinal sections of the bridging area showed that the mucosa was thin over the native muscle layer and it was then replaced by a layer of inflammatory cells towards the lumen [Figure 15]. Double staining with laminin and desmin revealed a thin basal membrane over some of the pericytes in the sprouting vessels in the bridging area and there were pericytes which looked as though they were about to detach from the vessel wall [Figure 16].
In the animals with significant leakage, a more aggressive inflammatory pattern was seen, with calprotectin-positive cells in the native muscle layers close to the bridging area and more, larger islets of CD3-positive islets in the bridging area.

In Paper IV, six piglets underwent surgery and lived for 20 days until sacrifice. They underwent surgery according to Method 2 in Paper 2. In the animals given Nexium (3-6), no signs of nausea or reflux were present. Two of six piglets had stent loss prior to 20 days, which gave rise to clinical signs of esophageal obstruction. At autopsy, the bridging area was easy to identify and retrieve in the animals with a retained silicone stent and the Biodesign mesh could be seen inside the bridging area. If the stent was lost, the adhesions between the bridging area and surrounding tissue were firmer and required sharper dissection for retrieval and the Biodesign mesh was lost together with the stent.
Histology from the bridging area of the piglets in which the stent was preserved had the luminal side covered with mature mucosa up to the edges of the native muscularis externa [Figure 17].

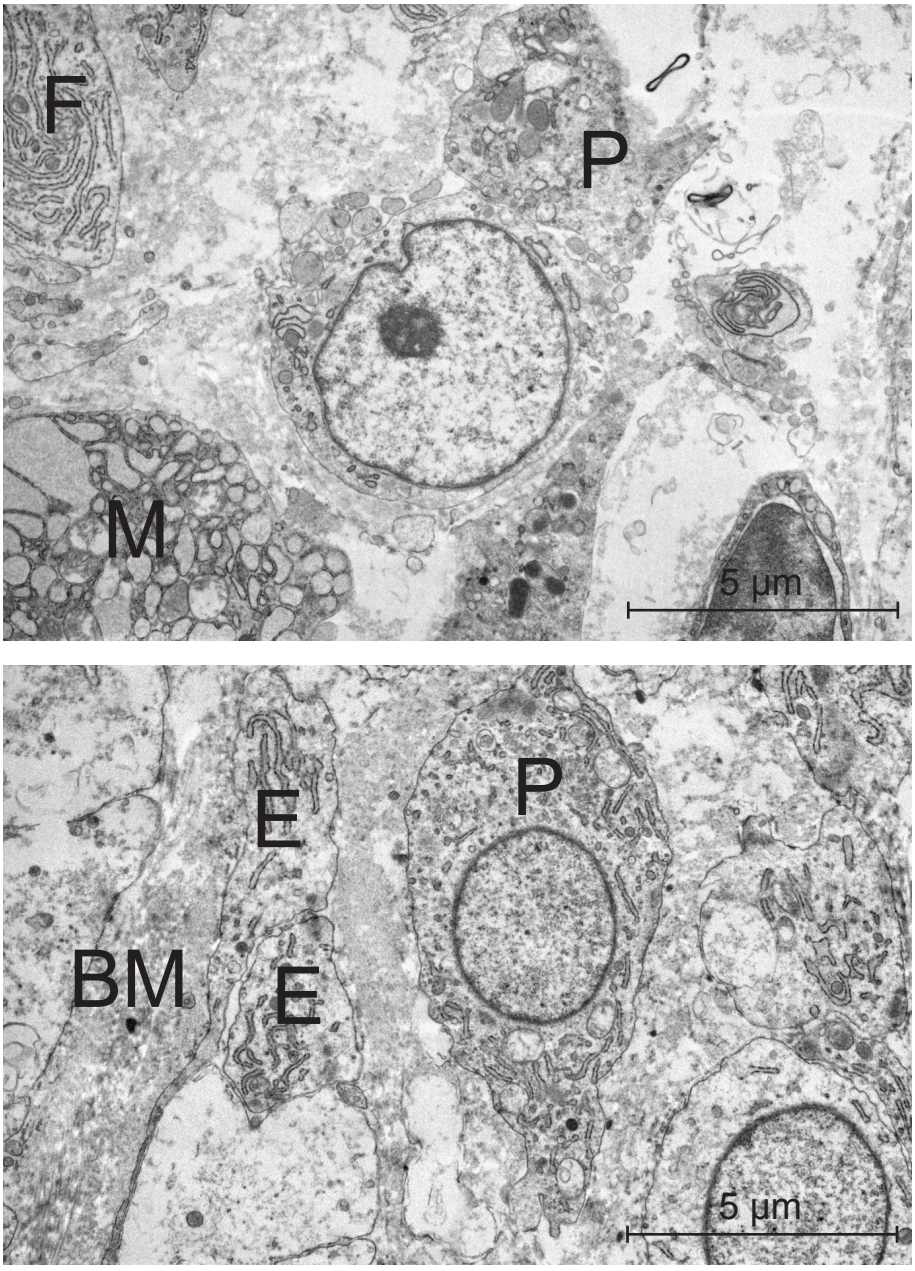

Figure 10. Electron microscopic images of the bridging area. A) In the center of the image a cell, almost devoid of organelles, is visible with the characteristics of an activated pericyte (P). In the upper left a probable fibroblast, rich in granulated ER, is seen (F). In the lower right a vacuole-rich macrophage (M) is found.
B) A sprouting vessel and the tip of endothelial cells (E) can be seen underneath a thick porous basal membrane (BM). On the outer rim of the BM a pericyte (P) is seen which seemingly is projecting towards the surrounding tissue.

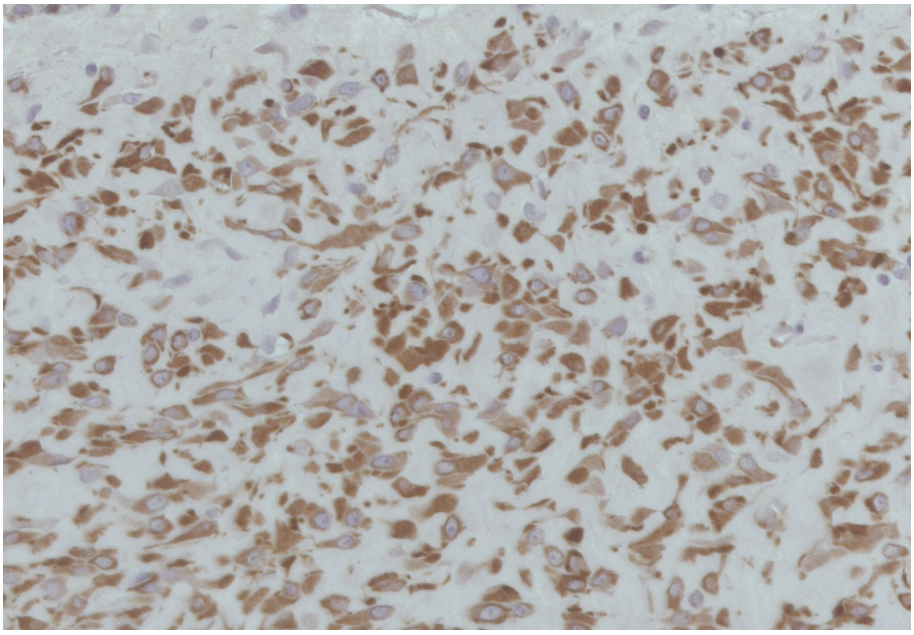

Figure 11. Paraffin section from the regenerating esophagus 4 weeks after operation. The section is processed to demonstrate desmin. Large cells often arranged in groups of 2 or more are seen. Bar = 50 µm.

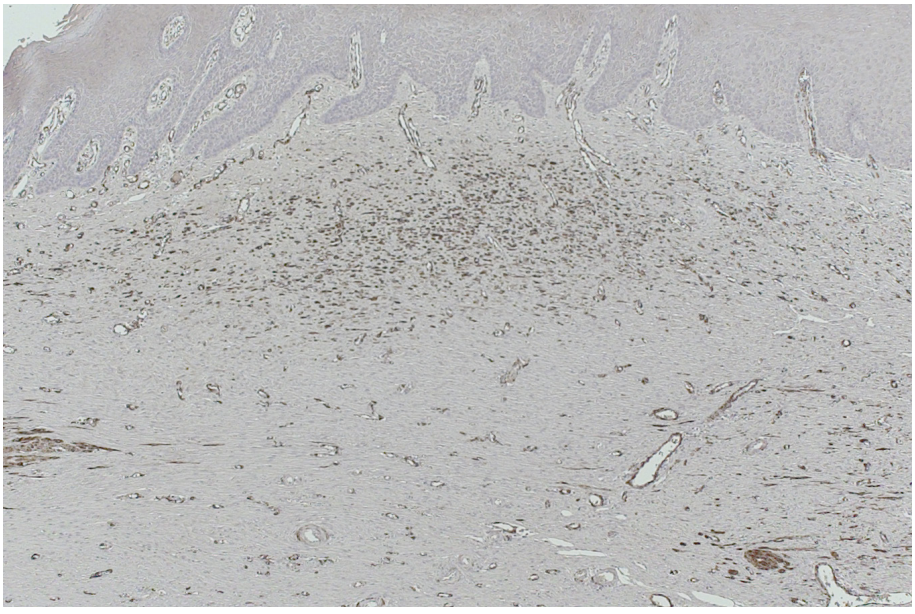

Figure 12. Paraffin section from the regenerating esophagus 16 weeks after operation. The section is processed to demonstrate desmin. A cluster of desmin-positive cells are situated in the lamina propria below the regenerated epithelium. Bar = 500 µm.

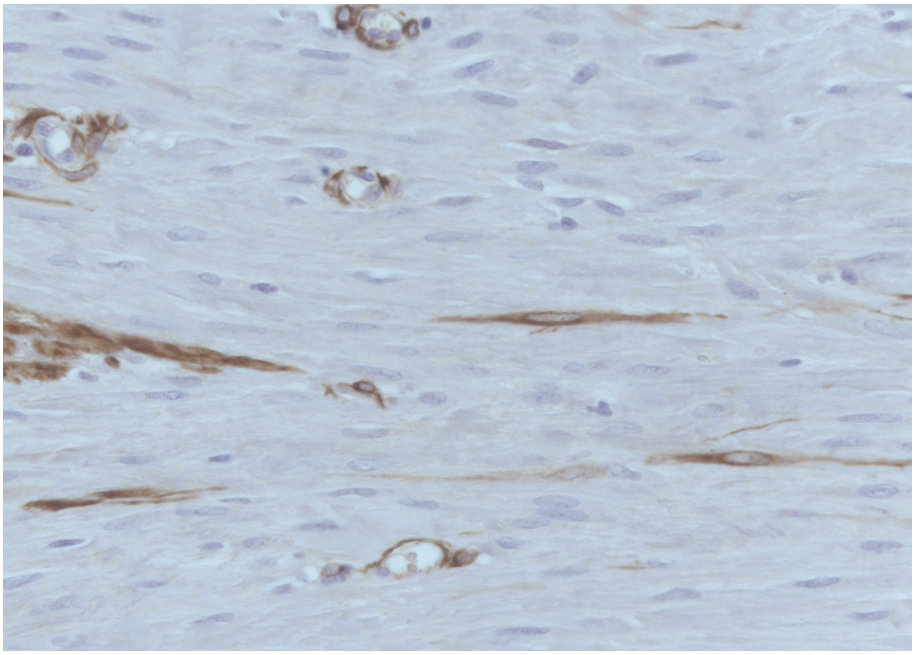

Figure 13. Paraffin sections from the edge of the bridging area 16 weeks after surgery. The section is processed to demonstrate desmin. Close to the cut, native esophageal muscle spindle shaped desmin positive cells are seen in the bridging area.

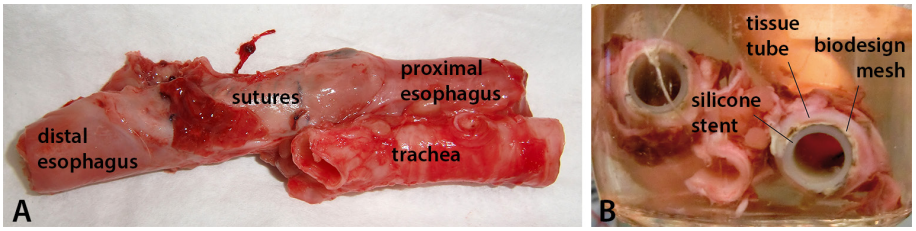

Figure 14. Macroscopic pictures of the bridging area at autopsy 9 days after surgery. A) Esophagus together with trachea is seen. The central white tissue is the bridging area, where the sutures can be seen under the newly formed connective tissue. B) The specimen divided in the center. Around the silicone stent, the Biodesign mesh can be seen under the newly formed tissue tube.

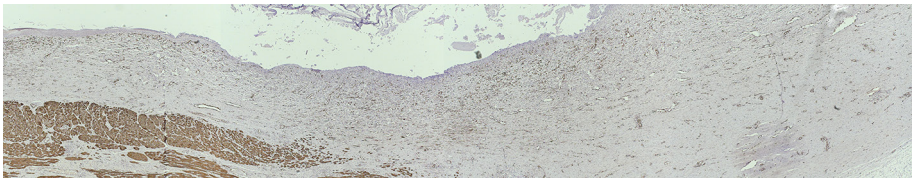

Figure 15. Longitudinal section from the proximal part of the esophagus 9 days after surgery. Desmin positive cells are stained brown.

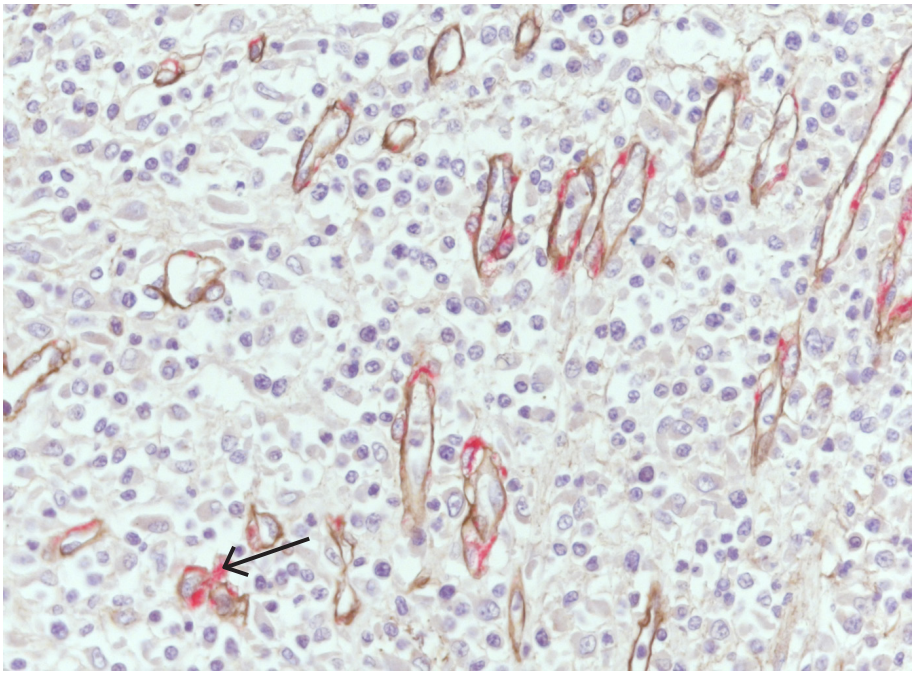

Figure 16. Section from specimen 9 days after surgery. Laminin, a basal membrane protein in the vessel walls, is stained brown. Desmin, which is present in the pericytes of the vessel wall is stained red. The arrow shows a pericyte, which seems to leave the vessel wall.

Figure 17. Longitudinal section from the proximal part of the esophagus 20 days after surgery. Desmin positive cells are stained brown. To the left the native muscle coat is seen. To the right is the bridging area, which contains smooth muscle cells organized in perpendicular layers. The specimen is approximately 1,2 cm long.

Central to the muscle edges, the mucosa became thin and was replaced by calprotectin-positive inflammatory cells. CD163 staining of the central portion of the bridging area revealed a rim of positive cells close to the lumen [Figure 18b]. Towards the adventitia, there was a layer of CD3-positive lymphocytes, which lay organized in strands and, further into the wall towards the adventitia, there was a layer of connective tissue where an abundance of new vessels and smooth-muscle, myosin-positive cells could be seen. In the center, smooth-muscle cells were organized in two perpendicular layers. The smooth-muscle cells were not evenly distributed but appeared in clusters throughout the entire bridging area. They were thicker and more organized in the center of the bridging area compared with the proximal and distal parts. Islets of calprotectin-positive cells and neutrophils persisted in the wall [Figure 19]. The center of the bridging area was stained for synaptophycin. In piglet 5, the vagal trunk could be seen on the outside of the regrown tissue. Close to the nerve, a small nerve was seen projecting into the outer part of the healing tissue, embedded in the regenerated layer of connective tissue and muscle cells. In this specimen, more smooth-muscle cells could be seen on the side of the wall where the vagal nerve was situated. No nerve penetrating the regenerated area could be seen in the remaining piglets.

In the piglet in which the stent was lost, staining for calprotectin revealed an abundance of positive cells throughout the entire wall of the bridging area. No CD163-positive macrophages were seen. As the bridging area appeared to contract, it was difficult to distinguish layers of CD3-positive lymphocytes and desmin-positive cells. The extent of mucosal regrowth was difficult to determine due to the poor organization of the specimen. However, there were cells positive for both desmin and smooth-muscle myosin in the wall of the bridging area.
CD163-positive macrophages in the bridging area were seen in all the piglets with the bridging graft in situ but could not be seen in the piglets in which the graft was lost. The difference did not become significant in terms of whether the stent/Biodesign was present or lost 20 days after surgery (P=0.07).
In comparison, CD163 staining of the nine-day specimen revealed that there were sparse CD163-positive cells next to the lumen in the piglets where no leakage occurred [Figure 18A,B].

The number of smooth-muscle cells in the center of the bridging area was more pronounced 20 days after surgery compared with after nine days (Fig 15 and 17). The CD 3-positive layer separating the regenerating muscle from the esophageal lumen had developed between nine and 20 days after surgery.

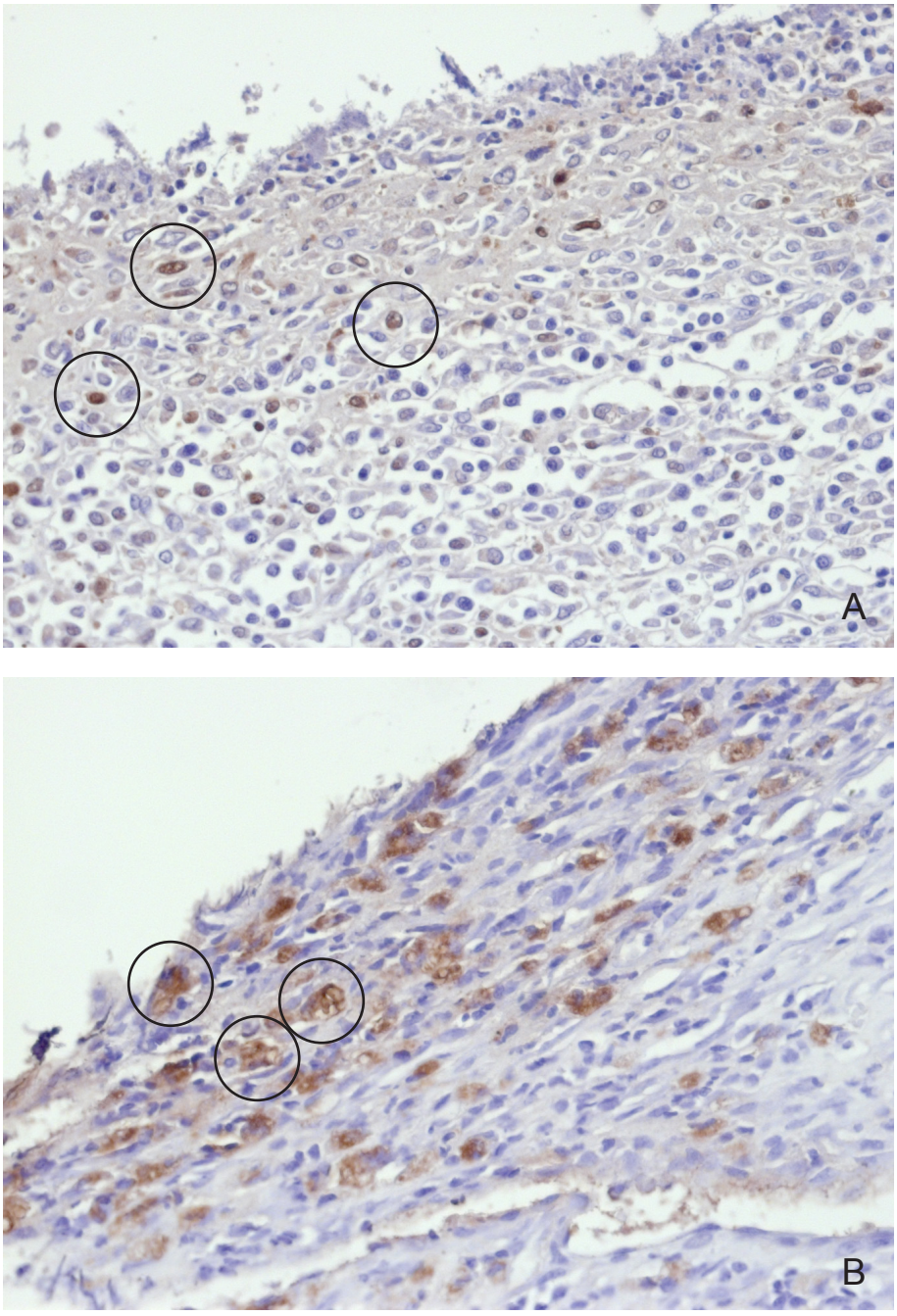

Figure 18. Sections stained for CD163 from the center of the bridging area A) 9 days after surgery B) 20 days after surgery. There is an accumulation of positive cells, close to the lumen at 20 days compared to 9 days after surgery. Some of the positive cells are encircled.

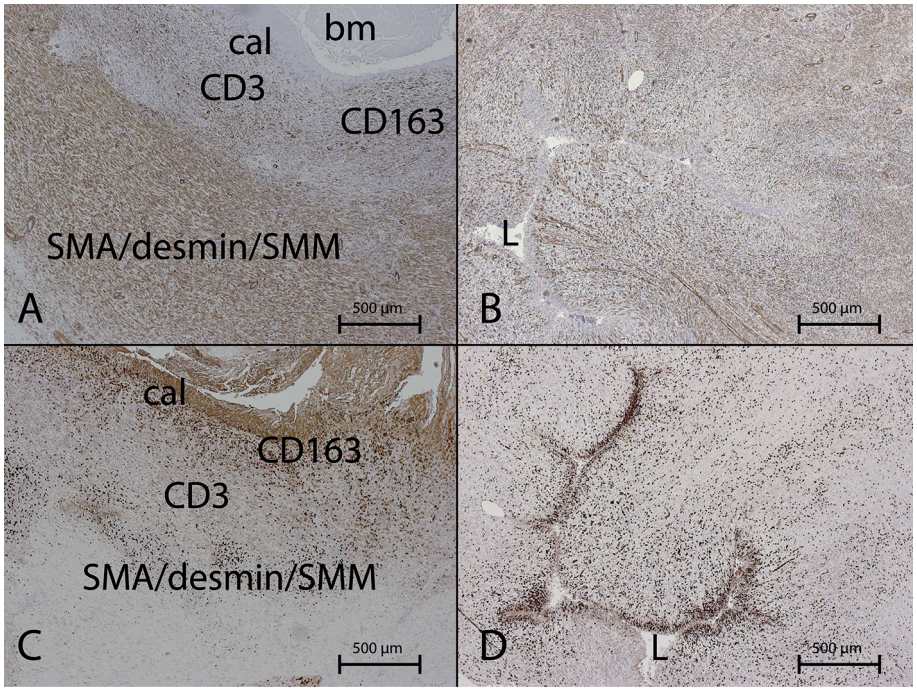

Figure 19. Transverse sections from the middle part of the regenerated area. A and C from an animal in which the stent was retained for 20 days; B and D from an animal in which the stent was lost. In A and C, the position of the main cellular components are marked in the picture.(bm = biomaterial, cal = calprotectin, SMA = smooth muscle actin, SMM = smooth muscle myosin)
A and B processed to demonstrate SMA. A) The wall of the regenerated area is organized in distinct layers. The distribution of the of the main cellular components are marked in the picture (bm = biomaterial, cal = calprotectin, SMA = smooth muscle actin, SMM = smooth muscle myosin). B) The wall show no obvious organization and the lumen in contracted. C and D processed to demonstrate calprotectin. Bars = 500 µm. Nuclei counter stained with hematoxylin

Table 1. Clinical features. (AV comm. = Atrio-ventricular septal defect, VSD = Venticle septum defect, volume in one second, PEF = Peak expiratory flow)

Pat. no	Assoc anomalies	Definite surgery type	Definite surgery (age, days)	Further surgical inventions (age in years) G = Gastro- intestinal P = Pulmonary GP=gastro and pulmonary symptoms
1	Mb Down, AV comm.	Delayed end to end	60	Reoperation due to leakage
2	Mb Down	Delayed end to end	156	
3	Single kidney	Delayed end to end	171	Collies gastroplasty (1)G Nissen fundoplication (2 2/12)GP, Pyloroplasty (7 7/12) G
4	VSD	Delayed end to end	115	
5		Delayed end to end	133	Nissen fundoplication (1 5/12) GP
6		Delayed end to end	229	Reoperation with resection and reanastomosis (11/12)G, Nissen fundoplication (1 1/12)G Take down of a previous fundoplication +myotomy+ pyloroplasty (9 5/12)GP
7		Collies gastro-plasty	144	Reoperation due to leakage
8	Urethral valve	Colonic inter-position	240	
9	Anal atresia	Delayed end to end	200	
10		Delayed end to end	150	Nissen fundoplication (2 5/12)G
11		Delayed end to end	81	
12		Shärli/Rao Gastro-plasty	133	
13	No inferior caval vein, VSD	Delayed end to end	150	
14	Apert syndrome	Delayed end to end	150	Nissen fundoplication (1 9/12)GP
15	Mb Down	Shärli/Rao Gastro-plasty	165	
16	Single kidney, VUR, hypo-thyreosis, hyperinsulinism, malformed ear canals	Colonic inter-position	67	

VUR = Vesico-urethral reflux, LLMI = Lipid-laden macrophage index, FEV1 = Forced expiratory

LLMI (age when BAL performed in years) **neutro i BAL**	**Summarized result of "spirometry"** **N=normal** **O=Obstrictive** **R=Restrictive**		**FEV1/PEF (A=no fistula, B=prox fistula)**	**LCI (SD)**		**Reflux index (%)** **Pathological:** **at 1 year of age >10%** **at 7 years of age >4%**	
	Age 1	Age 7		Age 1	Age 7	Age 1	Age 7
	No follow-up	No follow-up		-	-	89	5
	-	Technically incorrect	9,8 (A)	-	-1,2	3	2
48 (3 1/12) 20 neutro 43 (6 5/12) 12 neutro 21 (7 4/12) 6 neutro	-	O	7,0 (A)	-	13,4	14	30
	-	N	10,1 (B)	-	-0,3	38	10
	-	R	6,7 (A)	-	2,2	16	1
Food in airways (9 4/12)	-	R	6,6 (A)	-	3,9	0	0
LLMI No answer (1 yrs) 70 neutro (3 11/12) 18 neutro	-	O	7,8 (A)	-	1,9	28	0
	-	O	7,2 (A)	-	0,5	0	0
	O	N	10,6 (B)	-0,9	1,2	18	11
20 (2 yrs) 1 neutro	O	-	A	-0,3	-	2	-
LLMI < 10 (2 4/12 and 5 2/12) 2009 0neut	O	-	A	0,4	-	3	-
	N	-	B	1	-	57	-
	N	-	A	0,7	-	5	-
LLMI 50, 41 neutro (1 3/12)	O	-	A	3	-	16	-
	N	-	B	0,2	-	21	-
	Not evaluated	-	A	-	-	-	-

General discussion

Even though survival among patients born with LGEA is high (3,7), these patients still have a great deal of morbidity from both the airways and the upper gastrointestinal tract (43,44,45,62). Several authors have shown that problems from the airways, in the more common type of EA (Gross type C), originate to a large extent from the malformation rather than from gastroesophageal reflux with aspiration (56,61). Patients born with EA Gross type A, on the other hand, have been shown to have less involvement of the airways in the malformation (59,60). In our study, we found similar changes on spirometry examinations in patients with EA Gross type A, but the clinical manifestations appear to be attributable to gastroesophageal reflux with aspiration. MBW was within the normal range in 11 of 15 measurements (1 patient had 2 measurements) and three of four with an elevated LCI required surgery to prevent aspiration and progressive pulmonary damage. This implies that MBW might be a valuable tool for monitoring pulmonary function in these children.

According to the most recent clinical guidelines, gastroesophageal reflux disease (GERD) is defined as "reflux of gastric content in the esophagus which causes troublesome symptoms and/or complications" (82). In our study, all the LGEA patients have GERD, even if they are often asymptomatic due to adequate treatment. Other studies often report "Symptoms of GERD", followed by a lower incidence of GERD postoperatively (43,44).

Children born with EA are born premature and small for their gestational age. In patients born with EA Gross C, pulmonary morbidity affects the same patients who have growth retardation (89). Growth retardation is therefore probably a sign of morbidity rather than an innate part of the malformation. In LGEA patients, growth has been shown to be "very acceptable" (6) or normal (90). In our series, we noted that the patients were both light and short at birth and stayed that way until seven years of age. As most of our patients were within ± 2SD, we were able to claim that they were within the normal range, but, as a group, they did not catch up in height at all, only in weight. As LGEA patients have longer periods of hospitalization and more morbidity than the EA Gross type C, it is reasonable to suppose that their growth retardation is due to their morbidity. Future studies will show whether growth can be enhanced if morbidity can be reduced.

The terminology for children born with EA, where a primary anastomosis is difficult to achieve due to lack of tissue, is controversial. LGEA was initially only used for patients without a distal fistula (2,22,23). Other suggested abbreviations have been LGF (long-gap fistula), for patients with a distal fistula, and LGNF (long-gap non-fistula), for patients without one (91). In recent years, LGEA has been used for all patients in whom a primary anastomosis has been difficult to achieve (4,24,25). As the postoperative morbidity might differ, not only secondary to excessive surgery but also due to underlying differences in the malformed organs, it seems reasonable always to define the anatomic subtype according to Gross when reporting the different outcome measurements. Bagolan et al. showed that, among their LGEA patients, defined as all patients with a distance between the esophageal pouches longer than

three vertebral bodies, patients with Gross type A+B EA(55%) had a poorer outcome than the C+D patients, with more dilations and more problems with prolonged oral aversion (4).

In order to find a way to avoid some of the morbidity from which these patients suffer, we have developed a porcine model for studying guided tissue regeneration of 3 cm of the intrathoracic esophagus. Earlier work has shown that pigs are the most suitable animal for studying esophageal regeneration, as they have a muscle composition in their esophageal wall similar to that of humans (66,67,68). Children born with esophageal atresia always have smooth muscle in their distal esophageal segment (92,93). To our knowledge, the muscle composition at the bottom of the upper pouch has not previously been described. Replacement studies designed to help patients born with EA should therefore not be performed in dogs or rodents, as the results cannot be transferred due to the histological differences.

Since the beginning of the 20th century, it has been known that amphibians have an extraordinary regenerative capability in most regions of their body (94,95). Even though factors which account for some of this remarkable capability to heal have been elucidated (95,96), no one, to our knowledge, has been able to trigger a healing response of this kind in mammals. In the field of regenerative medicine, methods to achieve some sort of functional replacement of tissue after removal or injury have been studied. Esophageal replacements with ECM scaffolds have shown very good results when used to bridge partial resections of the esophageal wall, whereas stricture has been unavoidable when circumferential resection has been performed (8,9,10,97). The reason for this difference in healing response is not known. Our results suggest that the healing process has the ability to regenerate functional tissue, even after circumferential resections, but the process is sensitive and can easily be pushed towards scar healing. The M1/M2-macrophage paradigm, where the M2 phenotype enables regenerative healing, appears to work well when it comes to evaluating the healing process in the model. Factors which can be attributed to regenerative healing in the model could be mesh-muscle contact, ionic leakage over the created defect, nerve-macrophage interactions and much more. We are unable to tell whether any of these factors are important in our studies, but we plan to investigate this further.

Several authors have added cells to the implanted scaffolds in order to enhance the histological outcome (72,74,98). In a rodent study, where ECM was prepared from rat esophagi and seeded with mesenchymal stem cells prior to transplantation, muscular fibers were seen in the wall and the mucosa had healed over the graft after 14 days, when part of the cervical esophagus was replaced. The cells were enhanced with green fluorescent dye in order to be able to follow the fate of the seeded cells. When the implant was explanted after 14 days, no green fluorescence was detected (98).

When working on the replacement of the inferior caval vein in a murine model, it

has been shown that the mesenchymal stem cells improve the histological outcome by altering the inflammatory response in the graft rather than by being the building blocks of the reforming vessel (99). In our studies, the smooth-muscle cells in the bridging area might be recruited from the edges of the cut native muscle edges, but the morphology of the immature-looking cells in Paper II implies that it is the pericytes of the ingrowing vessels that are the cellular origin of the regenerated muscle. Double staining for basal membrane and pericytes in Paper III also supports the hypothesis that pericytes leave the vessel wall of the ingrowing vessels to become smooth-muscle cells in the esophageal wall. In vitro, it has been shown that a subset of human pericytes can be developed into several different tissues in the right organotrophic media (100). This implies that the scar healing, which is the most common end result after damage in humans, is not due to the lack of cells that can develop into functional tissue. If so, something else must be missing which prevents regenerative healing after damage.

In amphibians, where regenerative healing is common after damage, a certain amount of nerve to the damaged area is needed (95,96). Goodwin et al. recently showed that macrophages are another necessary component for limb regeneration in amphibians (101).
In our model, both leakage in the anastomosis early after replacement and stent/mesh loss affect the inflammatory response in the bridging area during the healing process. By changing the surgical method, we have been able to achieve patent anastomoses. Under favorable conditions, smooth-muscle cells accumulate in the neoesophageal wall and the mucosa grows from the edges towards the center up to 20 days after surgery. The bridging area is organized in separate layers with macrophages and neutrophils towards the lumen, with a layer mainly composed of strands of lymphocytes and vessels in the center. Further out towards the adventitia, a layer of connective tissue, including smooth-muscle cells, is seen. This organization implies that the tissue develops into more functional tissue than just a scar. The fact that the smooth-muscle cells organize into two perpendicular layers also implies that there is some reformation of the esophageal muscle wall.
Staining for an ingrowing nerve only revealed a possible sprouting nerve in one specimen after 20 days. This implies that either the healing process so far is nerve independent, or that the nerves are so sparse that they are easily missed when preparing the histological samples.
An ingrowing nerve is seen in one sample, M2 macrophages are seen in the samples with the best histological outcome and an increased amount of muscle is seen with time in our model. This suggests that we are studying a healing process similar to that seen in amphibians, i.e. the regeneration of functional esophageal tissue.
When the animals were given Nexium orally after surgery, all symptoms of nausea in the animals disappeared. Nexium might also be beneficial to the histological development of the bridging area, as it diminished the acid exposure of the area during the healing process. A longer follow-up time is needed to see whether this process ends up with the regeneration of functional esophageal tissue with time.

Strengths and limitations

The main limitations of the clinical study (Paper I) are that it is retrospective and that there are few patients all with a very complex medical condition. The strengths are that all patients with a gasless abdomen and a long gap between the esophageal pouches have been included and that they have been subjected to a structured follow-up protocol at one and seven years of age.

The main limitation of all the experimental studies (Papers II-IV) is the small sample size, which makes it impossible to identify factors beneficial to the outcome with statistical significance.
The strengths are that the animals are meticulously cared for and that we have been able to develop the methods to achieve better histological and clinical outcomes, with a fairly small number of animals.
As the follow-up time after surgery has been longer, the fast growth of Swedish pigs might be a problem, as the diameter of the silicone tube quickly appears to be outgrown. In the ongoing work in the porcine model, we hope to obtain a better estimate of how long a stent is needed to enable the optimal histological end result. If a longer stenting time is needed, the discrepancy in size between the stent and the native esophagus becomes too large. We then plan to examine the muscle histology in Göttingen minipigs, to see whether they are suitable for replacements studies with a longer follow-up time.

Conclusion and further studies

Patients born with LGEA suffer from morbidity due to gastroesophageal reflux. Clinically available methods for esophageal replacement carry a lifelong increase in the risk of demise and poor function of the esophagus. MBW might be a valuable tool in the follow-up of these patients, for the early detection of aspiration into the lungs, which warrants swift action to avoid progressive pulmonary damage.
Experimental esophageal replacement with bridging grafts ends up with stricture and scar formation when used to replace part of the thoracic esophagus. In our model, we can see the presence of remodeling, M2 macrophages and an accumulation of functional tissue in the replaced area 20 days after surgery. Further studies focusing on the inflammatory pattern in the regenerating tissue during different time frames after surgery might help us to improve the outcome and develop a method which can be used in LGEA patients.

Further studies

Further studies of the replacement of the porcine esophagus are planned in order to follow the histological development of the bridging area under favorable conditions and to further develop the fixation of the silicone tube so that it stays in place for as long as it takes to secure an optimal end result.
Functional tests of the explanted tissue in the bridging area are planned, in order to evaluate muscular activity.
The connection between macrophage phenotype, nerve sprouting, ECM exposure and regeneration is planned to be investigated in an amphibian model, in order to find ways to improve regeneration

Sammanfattning på svenska

Esophagusatresi (EA) är en missbildning av matstrupe och luftrör, som drabbar cirka 1 på 3000 födda barn. 10-15% av barnen har en missbildningsvariant där det är svårt att operera ihop de båda matstrupsanlagen. I de fall, cirka 8 %, där förbindelse mellan det nedre matstrupsanlaget och luftröret saknas, är det nästan alltid stor brist på matstrupsvävnad vid födseln. Idag opereras dessa barn efter tre månaders väntan, då matstrupen ofta vuxit till. Om det fortfarande fattas vävnad får delar av matstrupen ersättas med en bit tarm, en del av magsäcken eller hela magsäcken. Dessa behandlingsmetoder medför en ökad kirurgisk risk och medför dessutom livslång funktionsnedsättning i matstrupen.

Försök att ersätta delar av matstrupen i djurmodeller har pågått sedan 1950-talet. Genom att använda plaströr för att överbrygga defekter i matstrupen kunde man få ett rör bestående av ärrvävnad att bildas, som fungerade som passage mellan munnen och magsäcken. Då detta rör inte har någon muskelfunktion kom metoden aldrig att användas hos barn. De senaste årtiondena har istället försök gjorts med rör gjorda av bindväv från människa eller djur. Dessa ombildas helt till matstrupsvävnad om man ersätter en ”lucka” av vävnad, men om man ersätter en circumferent del så bildas ärr och den nybildade strupen växer ihop.

Ny forskning rörande hur kroppen läker när renframställd bindväv används i sår har visat att läkningen är beroende av det inflammatoriska svaret från kroppen. Framför allt har man sett att en viss typ av vit blodkropp, makrofager, kan ha olika uttryck på sin cellyta, och därigenom styrs hela inflammationen i olika riktning. Den klassiskt aktiverade makrofagen, som aktiveras av till exempel bakterier, underhåller inflammation och slutresultatet av processen blir ärrbildning. Om makrofagen istället blir aktiveras på ett annat sätt, kan det istället dämpa inflammation, och i flera modeller av skador/sjukdom kan kroppen då nybilda funktionell vävnad. Vi har i våra studier kartlagt resultaten för de barn som opererats för EA utan förbindelse mellan nedre matstrupsanlaget och luftrören under 15 år i västra delen av Sverige. Barnen opererades vid ca 5 månaders ålder. Ca 70 % kunde opereras med en skarv mellan det övre och det nedre matstrupsanlaget, 5 stycken behövde en förlängning av matstrupen med hjälp av en del av magsäcken eller en del av tjocktarmen. Alla barn överlevde, men alla lider av gastroesophageal refluxsjukdom, som gör att de måste äta magsyrahämmande mediciner dagligen eller opereras mot reflux. Barnen är små vid födseln och fortsätter att vara små under uppväxten.

Lungfunktionsundersökningar utförda vid 1 och 7 års ålder har gjorts och visade en påverkan på luftvägarna som sannolikt är en del av missbildningen. En ny typ av undersökning, ”Multiple breath wash-out” var normal hos flertalet av barnen, men hos de med förhöjt värde behövde 3 av 4(75%) opereras för att förhindra att mat kommer ner i lungorna. Denna undersökning förfaller vara av värde för att tidigt upptäcka de patienter som behöver en mer aggressiv behandling för att undvika lungskador.

I en djurmodell har vi studerat om en del av matstrupen hos växande griskultingar kan ersättas med ett biomaterial utvunnet av gristunntarm. När vi ersätter 3 cm av

matstrupen har vi kunnat se att det ersatta området kan utvecklas till ett vävnadsrör som binder ihop matstrupsändarna inom 10 dagar efter operationen. Vävnadsröret strukturerar sig i tre lager. Innerst ses två lager med olika typer av inflammatoriska celler. Ytterst ses ett lager med bindväv och ökande mängder muskelceller 20 dagar efter operationen. I dessa preparat finns rikligt med inflammationsdämpande makrofager. Om det läcker mellan matstrupens insida och utsida, eller om silikonröret som sätts in i matstrupen vid operationen släpper för tidigt, ändras den inflammatoriska bilden i vävnadsröret. De inflammationsdämpande makrofagerna försvinner och området ärromvandlas och skrumpnar. Ytterligare studier med längre uppföljningstid behövs för att utröna om den mer gynnsamma histologiska bilden kan fortsätta i området och vävnaden utvecklas till ny fungerande matstrupsvävnad. Om hypotesen stämmer skulle det kunna bli en metod som förbättrar funktionen i matstrupen hos barn födda med EA.

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