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A tissue-engineered artificial bile duct grown to resemble the native bile duct in a porcine model

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1. ABSTRACT

The aim of this project was to fabricate an artificial bile duct as a new potential treatment for biliary diseases. This project sought to evaluate a novel approach for repairing common bile duct injuries with a biosynthetic graft without an intestinal bypass.

2. SUMMARY

AIM: The reference standard technique for the reconstruction of the extrahepatic biliary tree is Roux-en-Y hepaticojejunostomy. This procedure is not devoid of complications and may not be feasible in some patients. This project sought to evaluate a novel approach for repairing common bile duct injuries with a biosynthetic graft. This allows the reconstruction of the anatomy without an intestinal bypass.

Methods: Study subjects were 5 pigs. Each animal underwent a biliary injury, using an open approach. A graft of a synthetic bioabsorbable polymer scaffold made of PHEA-PLA+PCL was used to repair the defect with absorbable sutures. Weekly liver function tests were performed. Animals were sacrificed at months three and six. Subsequently, gross and histological examinations were performed.

Results: There were no fistulas or abdominal collections. The polymer scaffold and, more specifically, the planar-shaped scaffold led to a regeneration of the bile duct. The histological examination showed lymphomonocytic infiltrate and neovascularization. In two animals, regeneration failed due to the collapse of the scaffold.

Conclusion: The use of polymeric scaffolds for the repair of bile duct injury may represent a new frontier in hepatobiliopancreatic surgery.

3. INTRODUCTION

3.1 Background

Laparoscopic cholecystectomy represents the gold standard in the treatment of cholelithiasis. The numerous advantages it offers in terms of outcome as opposed to the open approach have made it extremely widespread: less postoperative pain, shorter post-operative hospital stay, reduced risk of surgical wound infections, better aesthetic result and faster return to work[1]. Recent studies have shown that this procedure is however associated with an increased risk of lesions of the common bile duct (CBD), with incidence rates reported in the literature as high as 0.5-0.8%[1-3].

This rate is not negligible when compared with the total number of laparoscopic cholecystectomy procedures done in Italy, amounting to 47,456 according to the Italian Statistical Yearbook of the Ministry of Health for the first half of 2015.[4]

The treatment of iatrogenic lesions of the bile duct requires a multidisciplinary team, including, in addition to the surgeon, an endoscopist and an interventional radiologist. From a surgical point of view, open approach procedures have increased morbidity and mortality as compared to laparoscopic cholecystectomy and involve a worsening of patient quality of life[5-7]. Moreover, the survival of patients undergoing bile duct reconstruction following iatrogenic injury with current methods is lower than the survival of patients undergoing cholecystectomy alone, given that it appears to be correlated with both age and patient comorbidity. Moreover, patients undergoing bile duct reconstruction have an increased risk of developing cholangiocarcinoma[8].

At John Hopkins Hospital, Melton assessed the quality of life (QoL) of 89 patients undergoing surgery to repair common bile duct damage following videolaparoscopic cholecystectomy (VLC), comparing them with a group of 100 patients who underwent uncomplicated videolaparoscopic cholecystectomy and a group of 100 healthy patients. The results obtained showed a worse QoL in patients undergoing major surgery to repair CBD damage compared with two control groups, especially from a psychological point of view ($p < 0.05$)[6].

The misidentification of the structures present in Calot's triangle (**Figure 1**) represents the most common cause of bile duct injury, especially in cases related to operator

inexperience or anatomic variations.

The surgeon learning curve is a risk factor of biliary injury. Indeed, most injuries occur during the first 100 laparoscopic cholecystectomy procedures done by a surgeon. However, about a third of the iatrogenic injuries occur after performing even more than 200 procedures[9].

Misidentification of local anatomy represents the most frequent cause of iatrogenic bile duct injury, accounting for 70-80% of lesions according to some authors[10,11]. The laparoscopic manoeuvres that are most at risk of common bile duct injury are:

- Excessively deep dissection of the liver bed;
- Accidental resection of common bile duct or common hepatic duct instead of the cystic duct;
- Tear of the cystic duct or common bile duct (**Figure 2**);
- Thermal damage of the common hepatic duct or common bile duct (**Figure 3**);
- Damage to aberrant right hepatic duct mistaken for cystic duct;
- Lesion of the right hepatic artery mistaken for cystic artery;
- Thermal damage or injury due to clips on the right hepatic artery during haemostatic manoeuvres[12].

These manoeuvres can easily result in a lesion of the common bile duct due to the risk factors inherent in the laparoscopic approach[13]:

- Limitation due to two-dimensional vision;
- No manual palpation of the hepatic hilum to recognise individual structures;
- Loss of visibility during significant haemorrhage.

The literature has reported numerous anatomic variations of the biliary system[12] (**Figure 4**) and of arterial vascularisation in the gallbladder region[14] (**Figure 5**). Misidentification during the procedure may lead a surgeon, though expert, to cause accidental injury of the bile ducts. The cystic duct can have a spiral shape and cross the common bile duct anteriorly or posteriorly. The cystic artery can originate from the gastroduodenal artery or from the left or right hepatic artery.

The clinical history of cholelithiasis is very important in determining the risk of iatrogenic injury in the course of laparoscopic cholecystectomy. According to the 2012

guidelines of the EAES (European Association for Endoscopic Surgery), surgery must be as early as possible with respect to the onset of symptoms, within 48 hours, to ensure the best patient outcome and to reduce post-op mortality and morbidity[15]. A history of repeated episodes of acute cholecystitis and the presence of scleroatrophic cholecystitis are risk factors for bile duct injury in the course of the laparoscopic procedure[15,16].

In the event of intra-procedure bile duct injury, observed intraoperatively or in the post-op period, treatment may require different and complementary strategies, depending on the size of the lesion. Surgical repair may require solutions ranging from the simple positioning of subhepatic drainage to biliodigestive anastomosis in order to restore intestinal continuity in the most serious cases[15]. In all cases, these procedures involve reduced patient quality of life because they have a high level of morbidity (6.5%), and a greater risk of mortality (4.2%)[17,18].

In analysing the results of the treatment of bile duct injury after laparoscopic cholecystectomy in 88 patients, Stewart showed that only 17% of repairs carried out by surgeons in primary centres were effective, as opposed to 94% of success when the procedure was done in a tertiary centre by "dedicated" surgeons; similarly, the post-op hospital stay proved to be three times longer for procedures done in primary centres (222 days vs 78, $p<0.01$)[19].

The first approach is generally an attempt at end-to-end repair with positioning of Kehr's T tube (**Figure 6**). It has proven to be less effective than repair by direct suture, when possible, or Roux-en-Y hepaticojejunostomy (**Figure 7**) (63%). However, these and similar techniques are burdened by complications in the short and long term, such as cholangitis or recurrent stenosis[19-22].

In the case of significant bile duct injury, with possible loss of substance, the technique of choice is hepaticojejunostomy[19,23,24], although it has a high percentage of failure[25] and long-term complications, such as anastomotic stenosis and neoplasms in the extant bile duct, probably due to the onset of ascending cholangitis due to the elimination of the mechanism by which the duodenal papilla prevents reflux[26-28].

These considerations have driven the search for new solutions to treat common bile duct injury by using materials that can adequately replace the damaged common bile duct. In this scenario, regenerative surgery could represent a simpler and more

effective solution in the repair of iatrogenic damage of the bile ducts.

3.2 Regenerative medicine and tissue engineering

Regenerative medicine is an interdisciplinary field of research focused on the repair, replacement and regeneration of cells, tissues or organs to restore damaged functionality due to any cause, including congenital defects, diseases and traumas[29]. This field, therefore, comprises extremely diverse therapeutic areas, such as cell therapy and tissue engineering. "Cell therapy" is when the use of a scaffold is not needed, while "tissue engineering" is when a scaffold is necessary to support the regeneration of damaged tissue[30] (**Figure 8**).

Tissue engineering applies the principles of cell biology, materials science and biomedical engineering to create biological substitutes aimed at restoring and maintaining the normal function of sick and damaged tissues/organs (**Figure 9**).

In particular, tissue engineering, as a branch of regenerative medicine, uses biocompatible and completely resorbable natural or synthetic materials which do not give rise to adverse reactions by the host organism. These materials must be able to favour the regeneration of damaged tissue by providing a three-dimensional structure similar to the extracellular scaffold that supports the tissue regeneration process[31] which is affected by the microenvironment, as well as by the very characteristics of the scaffold (size, geometry, pore density)[32]. The scaffold provides support for the adhesion and implantation of cells which can be mobilised from the pool of endogenous cells, such as stem cells, promoting the growth of new tissue with the same morphological and functional features as the native tissue[33].

Although it is still an emerging field, regenerative medicine has already produced new therapeutic approaches, among which Apligraf, an engineered skin equivalent[34], and Osteocel, a regenerative bone therapy with adult stem cells[35].

Tissue engineering, as a branch of regenerative medicine, has provided a new flexible approach for the repair and regeneration of damaged tissues and/or organs and holds the promise of overcoming the limits of conventional therapies.

Different strategies can be applied for tissue regeneration: the seeding of cells on an in-vitro scaffold, the implantation of tissues grown in vitro on a scaffold, the implantation of a scaffold without cells to support in-situ tissue

regeneration. Regardless of the technique, the scaffold must feature a three-dimensional structure to support cell growth, as well as mechanical properties like those of the tissue, the regeneration of which it must guide (**Figure 10**)[36]. The characteristics of the scaffold are therefore of crucial importance in tissue engineering. In the design of our study, the scaffolds support *in-situ* tissue regeneration and provide the cells with mechanical support against the forces acting *in vivo*, while maintaining the mechanical integrity of the tissue during the early phase. In the late phase, its complete degradation does not further hinder growth. At the same time, it prevents the development of foreign body inflammatory reactions which can lead to the production of scar tissue or stenosis[31].

The attachment, proliferation, and differentiation of cells are strongly affected by the microenvironment associated with a scaffold, including the size, geometry, density of the pores[32].

The main properties of the chosen scaffold material are:

Biocompatibility: The scaffold must allow the migration, proliferation and normal function of the cells. It must not cause foreign body adverse reactions by the host. Its degradation products must not be toxic in vivo[37].

Biodegradation: The degradation rate of the material must be appropriate to the simultaneous production of new extracellular matrix by the host. A degradation time that is too fast may jeopardize the mechanical stability of the newly formed tissue; on the contrary, a degradation time that is too slow can hinder cellular proliferation and the formation of new tissue[38]. One technique used to control the degradation rate is the combination of several polymers with different degradation times, using different quantitative ratios of polyglycolic acid (PGA) and polylactic acid (PLA) in the formation of poly(lactic-co-glycolic) acid[39].

Mechanical resistance: The material must have a mechanical resistance similar to the native tissue in order to withstand the stress forces present *in vivo*. Its stiffness can be modified by varying the concentration of the polymer[40] or by combining it with other materials (e.g., a natural polymer)[41].

Porosity: The size and structure of the pores are responsible for cell homing, as well

as for the diffusion of nutrients and waste substances[42]. From this point of view, synthetic polymers have the advantage of greater structural modulation. For example, the centrifugation technique can change the size of the pores in the polycaprolactone (PCL) scaffold[43]. In general, pore dimensions must be comprised between 100 and 200 μm to allow appropriate cell movement, binding and the diffusion of paracellular soluble factors, intercellular signalling, the transport of nutrients and metabolites and in-vivo neovascularization[43].

In short, the "ideal" scaffold should have the following characteristics[44]:

- biocompatible and biodegradable, with a controllable degeneration rate;
- degradation products should be non-toxic and easily eliminated by the host organism;
- highly porous to facilitate oxygen, nutrient and waste transfer to ensure rapid vascularization and tissue in-growth;
- resistant to stress and deformation;
- not cause foreign body inflammatory responses.

The development of composite scaffolds by combining materials with different characteristics (**Table 1**) appears to be the most advantageous solution capable of meeting all the mechanical and physiological needs of the host tissue[45].

The biomaterials used to produce scaffolds can be divided into three classes:

- Materials of natural origin, such as collagen, alginate and fibroin;
- Decellularised tissue matrices (bladder and small intestinal submucosa);
- Synthetic polymers (PCL, PGL, PLA).

The materials of natural origin and decellularised tissue matrices have the advantage of biological recognition and low antigenicity, but their production is rather complex. On the contrary, synthetic polymers can be produced on a large scale with controlled properties (resistance, degradation rate, porosity).

Synthetic polymers have several advantages: the possibility of modulating the chemical, physical and mechanical properties, forming copolymers or mixtures with different monomers in the same polymer; porosity like that of natural tissues; presence of free chemical groups that can be used to incorporate other substances (e.g., heparin, antibiotics and growth factors) for direct bonding[46].

Synthetic polymers may be degradable or non-degradable in contact with biological fluids. Typical non-degradable polymers include: high-molecular-weight polyethylene, used in orthopaedics; polypropylene, used to produce prosthetic devices for abdominal wall surgery.

Biodegradable polymers (polyglycolic, polycaprolactone and polylactic acid) are generally aliphatic polyesters which degrade in a physiological environment due to non-enzymatic hydrolysis of the ester bond[47]. Degradation products are natural, non-toxic metabolites and are eliminated as carbon dioxide and water. Their biodegradation rate can be modulated by altering the crystallinity or the ratio between the polymers in the copolymer.

The interest of researchers has been concentrated in recent years on biodegradable polymers, such as polyglycolic (PGA), polycaprolactone (PCL) and polylactic acid (PLA) (**Figure 11**), for their applications in the field of surgery, pharmacology and tissue engineering (e.g., production of resorbable sutures, devices for bone structure fixation, carriers for the release of bioactive molecules, scaffolds for the regeneration of tissues or organs).

PCL is an aliphatic polyester obtained from the ring-opening polymerization of ε-caprolactone. Its good biocompatibility has favoured its use in tissue engineering, but it is not indicated for long-term applications due to its hydrophobicity and slow degradability in vivo. From a physical point of view, PCL is semi-crystalline with a melting temperature of 58-63°C and a glass transition temperature of about -60°C. It is elastic at room or body temperature.

It is easily processable and biocompatible and its mechanical and biodegradation properties can be modified by forming copolymers. It degrades slowly in vivo and has a high tensile strength and considerable elongation properties, which have allowed its use in various tissue engineering applications[48].

The degradation rates and physical and mechanical properties of PLA and PGA depend on the molecular weight or on their transformation in copolymers[49]. Since each polymer has its unique features, the development of composite scaffold, consisting of copolymers of two or more types of materials with different properties, is the most appropriate and advantageous solution for the production of scaffolds that are able to interact with the host tissue[45].

3.3 Preliminary studies in the literature on tubular scaffolds in common bile duct regenerative surgery

As regards the bile ducts, most *in-vitro* studies have focused on the formation of cell clusters similar to bile ducts in cell cultures of hepatocytes on collagen scaffolds; few studies have used synthetic scaffolds[50,51].

CBD reconstruction requires biomaterials having certain characteristics: biofunctionality, adequate degradation time, resistance to mechanical forces *in vivo* and to bile, and the ability to adapt to the rate of autologous regeneration to avoid bile fistulas and stenosis in the medium and long term.

Different grafts made of biological (collagen) or synthetic materials have been assessed in preclinical studies on animal models.

The results obtained with natural and autologous materials, such as collagen, taken from animal intestines have been unsatisfactory in the long term[52,53].

Rosen et al.[54] tried using porcine small intestinal submucosa to repair common bile duct injury, but the scaffold caused cicatricial sclerosis which developed into stenosis[55.] Although this material has the advantage of not causing foreign body reaction, it makes the recipient susceptible to zoonosis. Its use has been rather limited for all these reasons.

A tubular scaffold of porous collagen reinforced with polypropylene has been used to reconstruct bile duct defects in a canine model. The presence of polypropylene to ensure the necessary degree of resistance, however, reduced the flexibility of the CBD, increasing the rate of long-term complications[56].

ePTFe grafts have also been considered for the same purpose, using them both as patches and as substitutes of some sections of the biliary ducts; follow-up in the short term has nevertheless shown the development of segmental stenosis and partial or complete detachment of the prosthesis with the consequent formation of foreign bodies inside the biliary tree[57,58].

Tissue engineering, through the development of biocompatible and completely resorbable synthetic materials, has opened a new frontier in the regeneration of damaged tissues. The first successes were obtained in the reconstruction of blood

vessels[59] or the small intestine[60] by means of stem cell culture on polymer scaffolds.
A resorbable PGA tubular scaffold, interposed between the two stumps of the CBD, has been used in an experimental canine model without biliodigestive anastomosis. The graft favoured the growth of native tissues which reconstituted the anatomy of the CBD by forming columnar epithelium and extracellular matrix [61] (**Figure 12**).
The Saitama Medical University team (Miyazawa et al.) proposed the use of a polylactic acid and polycaprolactone copolymer reinforced with polyglycolic acid fibres (**Figure 13**) which was seeded with autologous bone marrow stem cells[62].
This scaffold which was used to make a bypass between the CBD and the duodenum (**Figure 14**) allowed the morphological and functional regeneration of the native bilioenteric tissue on the bypass six months after grafting.
There were no cases of biliary stenosis or fistulas. Similar results were also obtained in the control group grafted with the same polymer without the seeding of stem cells. These results suggest that the undifferentiated cells present in the peripheral circulation of the recipient can migrate to the graft implantation site, grow and differentiate regenerating new biliary tissue, like the native tissue, even after the synthetic scaffold has been completely absorbed and eliminated from the graft site[63].
The authors used the same scaffold to completely replace a 2-cm section of CD in a porcine model to assess the ability of the graft to repair a complete defect of the biliary tree. The histological exam at 4 months revealed the presence of columnar neoepithelium identical to the native tissue, demonstrating the ability to completely regenerate the bile ducts without causing stenosis, fistulas or foreign body reactions, considering the complete degradation of the prosthesis[64.65].
Other research groups are focusing on the creation of highly porous scaffolds that are able to favour cell grafting in the pre-implantation phase with stem cells and in the post-implantation phase by anatomical epithelial contiguity, as in the experimental study conducted by Zong et al. on porcine models using a “bilayered” PCL-PLGA biopolymer as scaffold (**Figure 15**)[66].
The research group of Eduardo E. Montalvo et al., using a porous tubular scaffold in collagen and ε-caprolactone in a porcine model, implanted a resorbable bioprosthesis to replace the CBD. Post-op follow-up was at 1, 3 and 6 months by magnetic resonance cholangiography, ERCP and choledoscopy. The follow-up images obtained by

magnetic resonance cholangiography at six months post implantation were satisfactory. The scaffold maintained a good seal (**Figure 16**) and there was evidence of the formation of neoepithelium on the inside (**Figure 17**). The histological and immunohistochemical results showed the presence of biliary neoepithelium and the formation of intramucosal glands in the neo-tissue[67].

3.4 Objectives

The objective of this study is to determine the ability of a bioresorbable scaffold, whether planar or tubular, to withstand the lytic action of bile and to result in the complete regeneration of a damaged bile duct in a porcine model.

In a preliminary phase, we will assess the behaviour of our scaffold in contact with bile. A necessary condition for the scaffold to act as a support for cell growth is, in fact, its resistance to the lytic action of bile which has specific characteristics. In the following phase, we will assess its ability to guide tissue regeneration of the damaged common bile duct by replacing portions of the bile duct with our material, in its planar and tubular forms. In an initial phase, the scaffolds used in the experiments should act as the native bile duct, ensuring an adequate flow of bile, in the absence of stenosis or leakage; subsequently, once degraded and absorbed by the host organism, they should be replaced by a neo-duct with anatomical and functional features that are superimposable to those of the native biliary tissue of the host.

4. MATERIAL AND METHODS

4.1 Our model

The polymeric scaffolds for testing were prepared in the Biocompatible Polymers Laboratory of the Department of Molecular and Biomolecular Sciences and Technologies (STEMBIO) of the University of Palermo.

The starting polymer, used to produce the copolymers, was α,β-poly(N-2-hydroxyethyl)-dl-aspartamide (PHEA). PHEA is a biocompatible synthetic polymer which has already been used as a drug carrier[68]. This polymer, combined with PCL (polycaprolactone) and PLA (polylactic acid), constitutes the base of the scaffold. PHEA-PLA+PCL is an elastic material with high mechanical resistance and good biocompatibility[69] (**Figure 18**).

It was obtained by electrospinning[70], a procedure that uses a high voltage source to charge a polymeric solution or molten polymer which is then drawn by a collector having the opposite charge, thus producing continuous biomaterial threads with a diameter of even less than μm. In our case, we applied a voltage of 6-7 kV and the biomaterial was emitted at a speed of 1 mL/h with a flight distance of approximately 20 cm (**Figure 19**). This method makes it possible to obtain planar or tubular scaffolds (**Figure 20**).

A study carried out previously by our group proved that this material has a good biocompatibility[74]. The study involved the use of six murine models with implantation of a PHEA-PLA+PCL scaffold in the muscle fascia of the back and sampling at 7, 15 and 40 days. At the end of the study, there were no episodes of rejection or complications, or cases of death related to the procedure (**Figure 21**)[74].

The project presented to the Ministry involved the use of at least 5 pigs weighing 40-45 kg each. During a procedure under general anaesthesia, they suffered a bile duct injury which was repaired by reconstruction with interposition of a tubular or planar PHEA-PLA+PLC scaffold.

All surgical procedures were carried out while maintaining the animal under adequate general anaesthesia (premedication: Zolazepam + Tiletamine 6.3 mg/Kg + Xylazine 2.3 mg/Kg - induction: Propofol 0.5 mg/Kg - Maintenance: Isoflurane + Pancuronium

0.07 mg/Kg). With the animal in a prone position and the 4 limbs secured to the operating table, the region of interest was shaved and the operating field was disinfected with povidone iodine 10%. After the surgical procedure, all the pigs received post-operative antibiotic treatment with oxytetracycline (20 mg/Kg a day for 3 days).

In post-operative period, the animals were monitored clinically and blood samples were drawn daily for the first seven days and then once a week to control the inflammation and cholestasis parameters. A follow-up ultrasound was performed at post-op day 7. The sacrifice of the animals was scheduled at three and six months, except in the case of a worsening of clinical conditions requiring a change of the scheduled deadlines.

In all cases, a microscopic histological examination of the grafted section was done to assess the response of the host and the degree of tissue regeneration, if any. In some cases, an immunohistochemical exam was done to search for specific endothelial markers by using Ig anti-CD31.

The study thus designed was initially submitted for approval to the Animal Welfare Body (O.P.B.A.) of the "A.Mirri" Institute (according to Italian Legislative Decree No. 26 of 4 March 2014 transposing Directive 2010/63/EU on the protection of animals used for scientific purposes). Following the meeting of 10/09/2014, it expressed a favourable opinion and on 09/10/2014 the project was submitted to the Ministry which issued its approval on 16 February 2015.

4.2 Experimental tests

4.2.1 Grafting of the scaffold in the gallbladder wall: biocompatibility test and resistance test to bile.

As already explained, one of our objectives was to assess the reaction of the scaffold to contact with bile. A necessary condition for the scaffold to act as a support for cell growth is in fact its resistance to the lytic action of bile which has specific characteristics.

We therefore assessed the ability of our scaffold to replace a portion of the gallbladder wall of approximately 2 cm^2.

After a median longitudinal incision (**Figure 22**), we accessed the hepato-duodenal region and isolated the gallbladder which, in pigs, is localised under the liver as in humans (**Figure 22 B**).

Once the gallbladder was identified and isolated, we clamped the gallbladder fundus and made an incision of about 2 cm^2 (**Figure 23 A**). We sutured a planar scaffold made of PHEA-PLA+PCL on the gallbladder by means of interrupted 5-0 Prolene stitches (**Figure 23 B**). A preparation of platelet gel was also applied on the polymeric patch with a platelet concentrate of $2x10^6/\mu l$ (about $8x10^9$ platelets in total). The gel was prepared on a "BIOFUGE STRATOS™" centrifuge. The production of PRP (platelets-rich plasma) was obtained by centrifuging the plasma: 1600 rpm for 12 minutes at 20°C. For the concentration of PRP and obtaining the PC (platelet concentrate): 4000 rpm for 5 minutes. The platelets were activated using calcium gluconate and BATROXOBIN using PLATELTEX™. Another scaffold patch measuring approximately 3 cm^2 was applied on the platelet gel and made to adhere to the gallbladder by the action of the gel, thus forming a sandwich patch. The remaining amount of 3 cc of platelet concentrate was also applied with a syringe (**Figure 24**). Finally, the surgical wound of the abdominal wall was sutured with non-resorbable type 0 stitches.

4.2.2 Cholecysto-jejunal tubular scaffold

The porcine model—a female of 23 kg of weight and 4 months of age—was premedicated and sedated as per the anaesthesia protocol described above. The hepato-jejunal region was accessed after a median longitudinal incision. The gallbladder was then isolated and a jejunal loop was mobilised. Both the gallbladder-scaffold anastomosis and the cholecysto-jejunal anastomosis were obtained by continuous suture with 5-0 slow absorbable monofilament. A plastic stent was also interposed to protect our scaffold (**Figure 25, 26**). Considering the quadrupedal position of the animal, we decided to parietalise the anastomotic loop (**Figure 27**) before closing the surgical wound to ensure greater stability of the anastomosis especially in the early post-op period.

4.2.3 Tubular scaffold on the common bile duct

We continued the trial on our porcine models by replacing a stretch of about 2 cm in length of the terminal part of the common bile duct with our polymeric tubular scaffold **(Figure 28)**, performing also a cholecystectomy to ensure maximum bile flow through our bile neo-duct (**Figure 29, 30**). During the procedure the CBD was identified and isolated. The common bile duct had a transverse diameter measuring < 4 mm. The technical difficulties linked to the small size of the common bile duct was overcome by using Prolene 7-0 sutures and binocular magnifying glasses. Both the proximal anastomosis and distal anastomosis were obtained by continuous suture. At the end of the procedure, there were no signs of leakage or bleeding and the anastomoses appeared to be well sealed. The scaffold had good wetting and showed no tension between the two anastomotic ends.

We closed the abdominal wall in layers and transferred the porcine model to the short-term observation centre.

4.2.4 Common bile duct patch

The porcine model—a female of 23 kg of weight and 4 months of age—received general anaesthesia as per the anaesthesia protocol described above. The hepato-duodenal region was accessed following medial longitudinal incision and we isolated the gallbladder and the cystic duct up to the common bile duct which measured approximately 7 mm in diameter (**Figure 31**). Considering the difference in size between the CBD (7 mm) and our tubular scaffold (4 mm), it was decided to make a wedge incision in the common bile duct and to place the scaffold as shown in **Figure 32**. We then sutured the scaffold with 6-0 Prolene stitches on the coledochotomy created in the common bile duct (**Figure 33**) and closed the peritoneum enveloping the common bile duct with continuous suture using 3-0 Polisorb (**Figure 34**). Layered closing of the abdominal wall.

4.2.5 Tubular scaffold on the left hepatic duct

After premedicating the female porcine model—4 months of age and 30 kg in weight—, anaesthesia was induced according to the protocol and we performed a xipho-umbilical incision and opened the parietal peritoneum. The gallbladder was

isolated and the course of the cystic duct to the point where it meets the right hepatic duct. The common bile duct was very short, just 1 cm in length from the bile duct confluence, and the left and right hepatic ducts were abnormal in length (**Figure 35**).
After isolating the two structures along a stretch of about 5 cm, a section of about 1 cm in length was removed by resection from the left hepatic duct. The PHEA-PLA+PCL tubular scaffold of about 1 cm in length was positioned and the two ends (proximal and distal) were closed by semi-continuous suture with 6-0 Prolene (**Figures 36, 37**).
Considering that the broad dissection needed to correctly view the right and left hepatic ducts did not allow reperitonealization, omental fat was placed in the hepatic hilum. After isolating and detaching the small omentum and checking the haemostasis of the short gastric vessels, the latter was used to coat the neo-duct with a spiral pattern (**Figure 38**).

5. RESULTS

5.1 Scaffold grafting in the gallbladder wall

The porcine model survived the procedure and had no complications related to the positioning of the scaffold (leakage or abdominal collections). Antibiotic therapy with oxytetracycline (20 mg/Kg/day) was administered for 3 days. A liquid diet was started after 12 hours and a solid diet after 24 hours. In the first week, daily samples were drawn to check the cholestasis parameters which were all within range. At one month from the procedure an exploratory laparotomy was performed for a macroscopic assessment of the scaffold conditions (**Figure 39**). The procedure showed an intense inflammatory reaction which required thorough lavage. The abdominal exploration did not however show intra-abdominal collections or indirect signs of leakage. The macroscopic exploration showed an intact gallbladder, without signs of inflammation in progress. The portion of tissue on which the scaffold was implanted could not be distinguished from the surrounding tissue and therefore it was decided to postpone the sacrifice of the animal to three months. Over the three-month period, the animal maintained good clinical conditions and gained about 20 kg of weight. After the agreed three-month period, the animal was sacrificed and a cholecystectomy was performed. Macroscopically, the structure of the scaffold appeared to be indistinguishable from the gallbladder, but there was an intense adhesion reaction.

The histological study of the porcine gallbladder was carried out at the Istituto Zooprofilattico by Dr Puleio, the designated anatomic pathologist of the research project. After fixation and reduction, the samples were processed as done routinely for paraffin embedding. Serial sections (5 μm) of each sample were stained with hematoxylin-eosin (H&E) (**Figures 40, 41, 42, 43**).

In **Figure 40,** it is possible to observe the completely reconstituted mucosa of the gallbladder, coated by simple columnar epithelium with developed villi, while, below it, past the gallbladder wall-scaffold wall interface (green arrow), there was widespread monocyte infiltration.

The other histological sections showed chronic inflammatory infiltration in the portion

of the scaffold (lymphocytes, macrophages and giant cells). The inflammation was due to foreign body reactions. The neoangiogenesis component is present and in this phase it probably supports the intense inflammatory response determined by the scaffold.
Thanks to the collaboration of Prof. R. Alessandro, Department of Biopathology and Medical and Forensic Biotechnologies, the scaffold sample was examined by immunofluorescence using anti-CD31 monoclonal antibodies. The sample was embedded in formalin, treated with 30% sucrose, included in OCT (antibodies) and stored at -80°C. Part of the sample was cut into slices with a thickness of 8 μm in a cryostat for subsequent IF and analysis under confocal microscopy (**Figures 44, 45, 46 and 47**).
Staining revealed the presence of numerous vessels of different size around the fibrous tissue. Serial imaging along the *z* axis allowed us to determine the correct organization of the vessels and confirmed the good response in terms of neoangiogenesis at the level of the scaffold. The electronic microscope analysis of a residual fragment of the scaffold showed its structure with the presence of some cellular elements (**Figures 48 and 49**). The fibrillary polymeric structure of the scaffold appeared to be preserved in several points (**Figures 50 and 51**).

4.2.5.2 Cholecysto-jejunal scaffold

The postoperative course in the early days was regular. The animal model had no fever, had normal bowel movements with stools of normal colour and the blood exams did not show any increase in inflammation and/or cholestasis parameters. On post-op day 7, we performed an abdominal ultrasound which was normal, without any evidence of abdominal collections. During a clinical examination at one month post procedure, the patient had no fever, had normal bowel movements and showed a net increase in body weight (7 kg). Blood exams showed no signs of anaemia or any increase in inflammation or cholestasis parameters. Considering the good clinical conditions, it was decided to schedule the sacrifice of the animal at six months post procedure.
During the sacrifice of the animal, we found no signs of neogenesis of the bile neo-duct as we expected. In light of an almost complete resorption of the scaffold, there was evidence of a foreign body granulomatous reaction with the obliteration of our

anastomoses and the passage of bile through its natural duct. During the procedure, no pericholecystic fluid collections or signs of perforation were found. The scaffold was completely absorbed and replaced by granulation tissue and fibrosis which obliterated the anastomotic stumps on both the gallbladder and jejunal side (**Figure 52**). The failure of the neo-duct to form and complete resorption of the scaffold did not make it possible to carry out a final histological exam.

5.3 Tubular scaffold on the common bile duct

The post-op clinical course was normal. The animal fed regularly, had no fever and had regular bowel movements with stools of normal colour. The follow-up blood tests in the first two weeks were regular. Considering the good clinical conditions, we transferred the animal to another site at one month after surgery to continue postoperative hospitalization. The animal died due to cardiocirculatory arrest during the transfer. In order to carry out a macroscopic and microscopic analysis, we froze the animal, with all its limits.

The autopsy did not show the presence of any intra-abdominal fluid collections or signs of bile peritonitis, and we were able to see that the scaffold and anastomoses at 1 month after the procedure still held (**Figures 53, 54, 55**).

The operative piece was then examined microscopically. After fixation and reduction, the samples were processed as done routinely for paraffin embedding. Serial sections (5 μm) of each sample were stained with hematoxylin-eosin (H&E). This exam revealed the presence of the scaffold consisting of amorphous material partially detached from the mucosa (**Figure 56**). In one area, there were clear signs of partial integration (**Figure 57**).

5.4 Common bile duct patch

During post-op week 1, the porcine model had no fever, inflammation and cholestasis parameters were normal and stools had a normal colour. The animal fed regularly and as per the protocol we performed a follow-up ultrasound which showed no signs of fistula or abdominal fluid collections. The animal was then taken to another facility to continue hospitalisation.

Considering that the procedure did not provide for the creation of a bile neo-duct, but only the repair of a stretch of about 2 cm of CBD on its front side, we decided to sacrifice the animal at one month after the procedure. We found macroscopic evidence of the complete integrity of the CBD and of the absence of the scaffold adhering to the latter (**Figure 58**). We therefore performed a choledochotomy and explored the CBD. The scaffold was found fluctuating therein and was partially degraded.
Electron microscopy (**Figure 59**) confirmed only the partial degradation of our scaffold which after a month still showed an intact fibrillar structure in many of its parts.

5.5 Tubular scaffold on the left hepatic duct

The postoperative course in the early days was regular. The animal model had no fever, had normal bowel movements with stools of normal colour and the blood exams did not show any increase in inflammation and/or cholestasis parameters.
The follow-up ultrasound at about post-op day 20 showed a dilation of the left hepatic duct (**Figure 60**).
The porcine model had no jaundice or hypochromic stools. The animal was sacrificed one month after the implantation of the scaffold. After lysis of numerous adhesions, the cholecystocholedochal region was reached and was devoid of intra-abdominal fluid collections. The scaffold was detached from the left hepatic duct in its distal portion, while it was still anastomosed on the proximal end (**Figure 61**). The intense inflammatory reaction on the two sides of the anastomosis resulted in the obliteration of the duct, which therefore explains why there was no bile leakage.

6. DISCUSSION

The objective of this study is to determine the ability of a scaffold to withstand the lytic action of bile and to result in complete regeneration of a damaged bile duct in a porcine model.

Therefore, in our first experiment we grafted a portion of planar scaffold in the wall of the gallbladder. The results obtained in this first phase showed that the scaffold is able to replace a portion of bile tissue. Both during the experiment and in the subsequent re-laparotomy, there was no evidence of leakage or complications, such as abdominal fluid collections. Moreover, microscopic analysis at three months showed a progressive degradation of the scaffold and a concomitant regeneration of the bile neo-tissue, as shown by the presence of bile neo-epithelium in the implantation site under optic microscopy.

The inflammatory stimulus appeared to be the trigger of tissue regeneration, as is clear from the abundant lymphatic and monocytic infiltration. Further confirmation of the significant regenerative stimulus induced by the scaffold was provided by the extensive process of neoangiogenesis shown both under optical microscopy and by immunofluorescence. The scaffold triggered the formation of neo-endothelium and neo-vessels that were organised along the correct spatial axis and functioning.

Our material proved to be resistant to the lytic action of bile, as well as conducive to good tissue regeneration, though limited to a small area of only 2 cm^2.

These considerations encouraged us to use our tubular scaffold in an effort to obtain the complete regeneration of the CBD in all its thickness. Obviously, the bile flow conditions to which a tubular scaffold is exposed in the common bile duct are completely different from those of a planar scaffold in the gallbladder.

The low bile flow to which the scaffold is exposed in a cholecysto-jejunal anastomosis was probably responsible for its collapse.

In light of an almost complete resorption of the scaffold, there was evidence of a granulomatous foreign body reaction with the obliteration of our anastomoses and the passage of bile through its natural duct. We attributed this result to the failure to ligate and to close the common bile duct during the experiment. This technical decision that was made pre-operatively to reduce the risk of post-operative perianastomotic leakage probably resulted in a low bile flow through the anastomosis and hence in the collapse

of the neo-duct constituted by the tubular scaffold. During the procedure, no pericholecystic fluid collections or signs of perforation were found. The scaffold was completely replaced by granulation tissue which obliterated the anastomotic stumps on both the gallbladder and jejunal side. However, the presence of the scaffold prevented the formation of biliary and/or enteral fistulas.

Based on these considerations, we therefore decided to perform an anastomosis between our tubular scaffold and the common bile duct to ensure maximum bile flow inside of the scaffold.

However, the premature death of our animal model did not allow us to obtain final results. The procedure was successful from a "technical" point of view and the death is not attributable to a complication due to our procedure. This finding, confirmed by the good postoperative course during the first month, was further supported by the lack of intra-abdominal fluid collections during the autopsy. The microscopic examination of the surgical specimen was not able to determine the complete integration between the native tissue and the scaffold, due to the short time that elapsed between the procedure and the death of our model. The degradation rate of the material of which the scaffold is composed is approximately 6 months, as also shown by previous experiences. Therefore, a month is not enough time for the complete degradation of our scaffold and its replacement with a neo-duct. However, there was partial integration in some points, thus signalling the initial stages of regeneration.

It should also be pointed out that the scaffold fulfilled its biological “task”, allowing normal bile flow, without any biliary fistulas or stenosis, as confirmed by the macroscopic and postoperative exams. As explained in the introduction, the scaffold is a support which, by acting as the tissue it replaces, allows the migration of cells and their differentiation into a neo-tissue up to its degradation. In this case, we were not able to see a regeneration of the tissue. Nonetheless, the scaffold certainly fulfilled its "structural” task, thus confirming its biofunctional properties.

The biofunctional properties of the scaffold, its structural resistance to bile and its ability to induce tissue regeneration in the host is further confirmed by the experience with the patch on the common bile duct. In this case, the smaller size of the defect probably led to a faster regeneration of the damaged CBD, (compared with the formation of a neo-duct) causing a rapid repair of the biliary tissue compared with the

degradation rate of our scaffold (six months). In this case, we were able to identify a macroscopically intact common bile duct, without signs of discontinuity. The lack of prior abdominal fluid collections or signs of biliary fistula confirmed that our scaffold is able to resist bile and to be watertight, both on the anastomotic side and inside the central structure.

The greater ability of the planar version to regenerate damaged tissue compared with the tubular version was confirmed by the experiment involving the interposition of the scaffold in the left hepatic duct. From the very beginning of the experiment, this model posed technical challenges linked to the abnormalities of the biliary tree. The animal had a shorter than average common bile duct after the confluence of two hepatic ducts of abnormal calibre and length. This condition required an anastomosis between the left hepatic duct and the scaffold that posed considerable technical difficulties. Perhaps, in the presence of smaller than average ducts our scaffold lacked sufficient bile flow to ensure patency, thus resulting in the collapse of the tubular structure, as was the case of the cholecysto-jejunal scaffold. However, in this case as well, the scaffold resulted in granulation tissue, which, though not forming a neo-duct because of the collapse of the tubular structure, avoided the onset of a high volume biliary fistula.

Therefore, the planar version of our material showed a good ability to regenerate damaged bile tissue, while, as regards the ability of the tubular version to guide the regeneration process of tubular structures as complex as the common bile duct, further technical adjustments are needed, although the preliminary results give us reason to be cautiously optimistic.

In fact, the material composing the scaffold has proven to be able to resist the lytic action of bile. Moreover, its three-dimensional structure allowed performing a technically complex anastomosis, with calibres as small as 4 mm. From a technical point of view, the material ensured a good seal of the anastomosis and in all the experiments the scaffolds proved to be easily handled thanks to their mechanical properties.

The PHEA-PLA has homogeneous fibres with morphological characteristics like those of the native ECM. The combination by elettrospinnig of PHEA-PLA with PCL allowed us to obtain a copolymer with good elasticity and mechanical strength,

probably due to the greater regularity and homogeneity of the size of the constituent fibres.

The elastic properties and microporous structure are responsible for two other results of our models: the total absence of bile leakage through the wall and excellent resistance to the passage of the suture needle which ensures a perfect seal of the anastomosis. The ultra-structure of the scaffold with an intricate web of electrospun nanofibers, distributed in a space just 0.5 mm thick and forming pores with an average diameter of about 150 μm, allows cells to colonise the graft, while preventing fluids to exude from the surface. These are the same features that allow the tissue to remain intact despite the passage of the suture needle, since the fibres composing it spread apart and are not damaged or sheared by the mechanical action of the suture.

The material has also proven to be perfectly biocompatible, and is resorbed thanks to the inflammatory reaction of the host tissue. This inflammatory reaction was characterized by the presence of diffuse lymphatic and monocytic elements and polynucleate giant cells, as the histological cross-sections demonstrated. Both the microscopic analysis and the immunohistochemical analysis confirmed the presence of significant neoangiogenesis. The formation of neo-vessels is the essential element for the regeneration of tissue because it determines the ability of the circulating elements to reach the implantation site, facilitating cell homing. These results are all the more significant if one considers the tissue in which it occurred. In fact, bile, unlike blood, carries no cellular elements capable of repairing tissue. Therefore, the scaffold favoured the inflammatory reaction of the host and performed its task in supporting the lymphatic and monocytic infiltrate and the neo-vessels.

Our tubular scaffold is 1 mm thick, thus proving to be optimal in withstanding mechanical stimuli *in vivo*, as demonstrated by the absence of early postoperative complications; however, the excessive "compactness" of the scaffold perpetuated the chronic inflammatory phenomena of stenosis due to its slow degradation rate. The advantage of synthetic polymers is also linked to the ease of producing them on a large scale and easily modulating their physical and dimensional properties. Therefore, in the further studies we will conduct we can use thinner scaffolds. The ideal thickness seems to be 0.5 mm, like those of the planar structures used, which have provided the best results. The possibility of repairing biliary injury with loss of substance through

the use of synthetic planar patches is currently not a technique reported in the literature, precisely because of the lack of devices with optimal characteristics. In our experimental models, the planar scaffold has always resulted in the regeneration of tissue in the absence of leakage and complications in the short and long term, thus favouring the complete healing of CBD damage, without anatomical or functional sequelae.

Therefore, these materials could pave the way to new technical applications in the field of hepatobiliary and pancreatic surgery.

7. CONCLUSIONS

The scaffold used in our study differs from others so far used and described in the literature. The PHEA-PLA+PCL biomaterial seems to have characteristics that make it suitable to be a temporary substitute for the biliary tree for all the characteristics described above.

The perfect biliary prosthetic material, in fact, should meet some fundamental requirements:

- Be biofunctional;
- Be biocompatible;
- Have an adequate degradation rate to allow complete replacement of the scaffold once biliary neo-tissue is regenerated;
- Have good mechanical resistance;
- Not cause stenotic phenomena which may be responsible for the onset of obstructive jaundice.

Our study succeeded in demonstrating all the objectives mentioned above except for the possible presence of long-term stenotic phenomena for the tubular scaffolds. Probably an excessive "compactness" of the scaffold lengthens absorption time, thus perpetuating the chronic inflammatory process causing stenosis and/or the rejection of the scaffold. In future experiments, we will use thinner and more porous scaffolds that can ensure better colonization of the scaffold by the cells around the CMD and less inflammatory phenomena in the long term.

By contrast, the planar scaffold has proven to be able of guiding and favouring complete regeneration of damaged tissue. These preliminary studies give us hope that we will be able to develop future devices that can be used in clinical practice in humans as well.

8. TABLES AND FIGURES

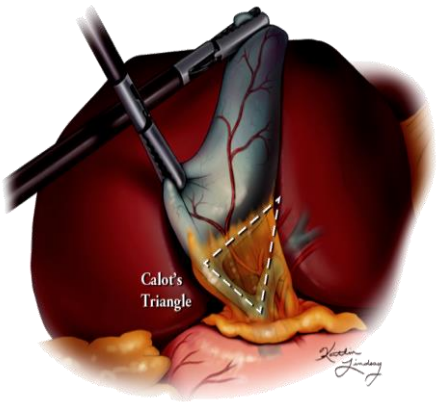


Figure 1. *Calot's Triangle*

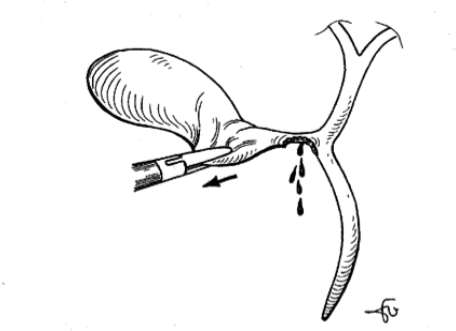


Figure 2. *Tear of the cystic*

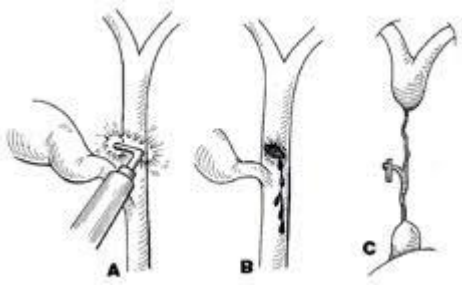


Figure 3. *Thermal damage of the common hepatic duct or common bile duct.*

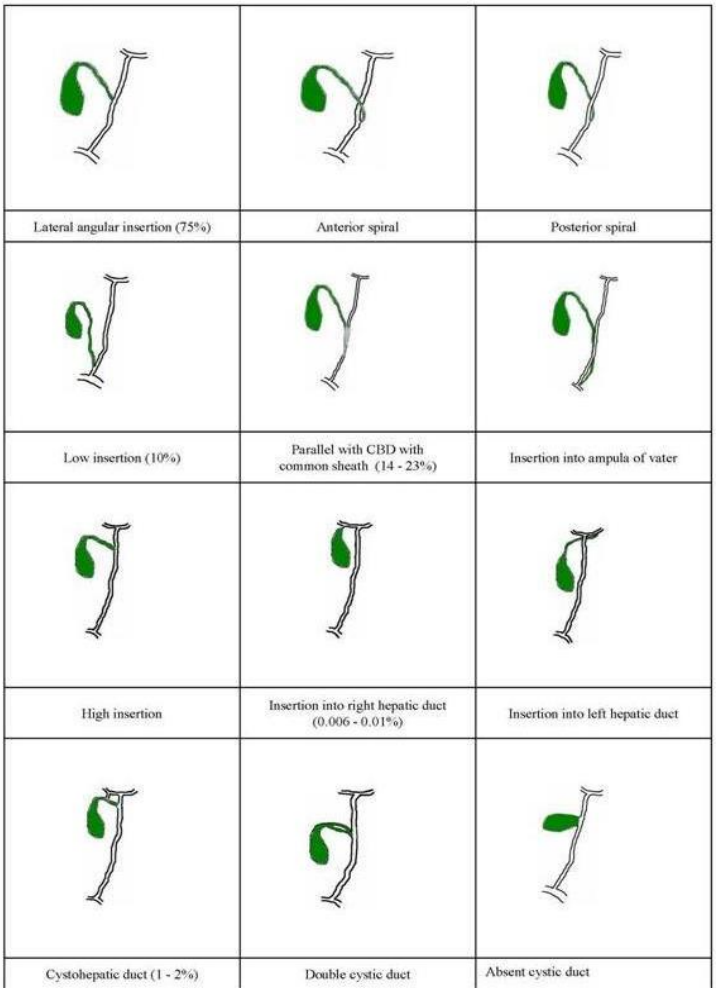


Figure 4. *Congenital abnormalities of the cystic duct*[10]

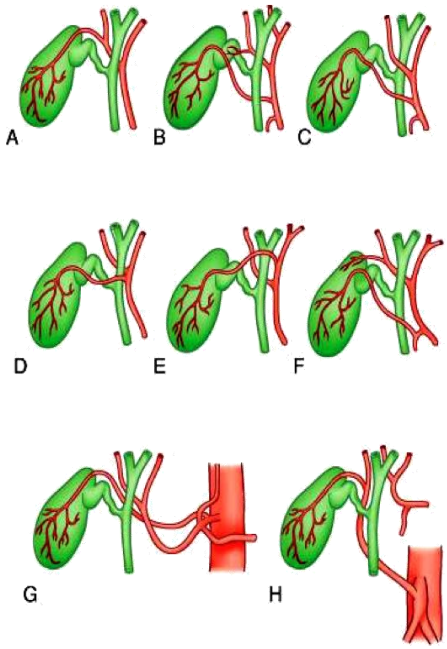


Figure 5. *Main abnormalities of the cystic artery*[11]

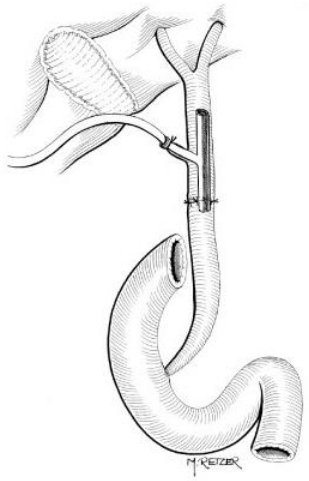


Figure 6. *Kehr's T Tube*

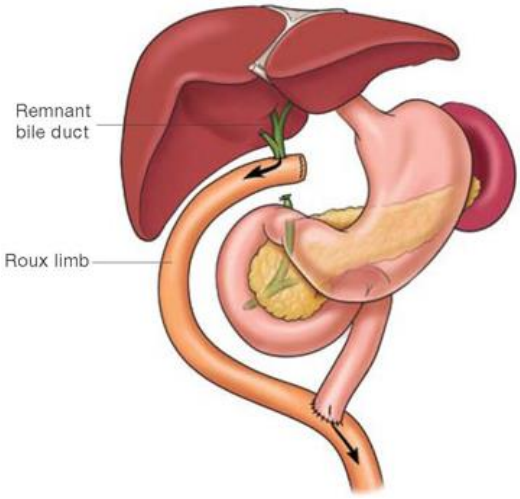


Figure 7. *Roux-en-Y hepaticojejunostomy*

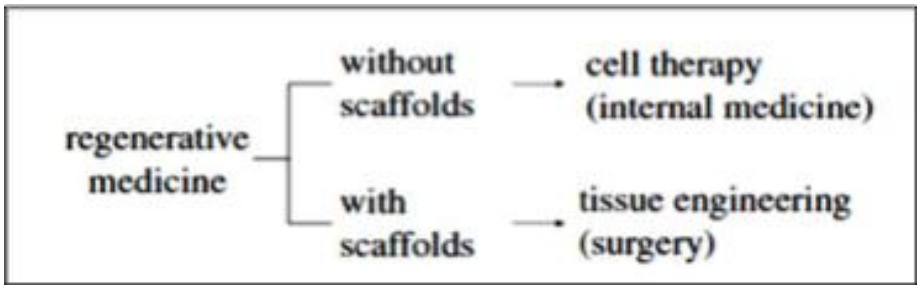


Figure 8. *Regenerative medicine*[40]

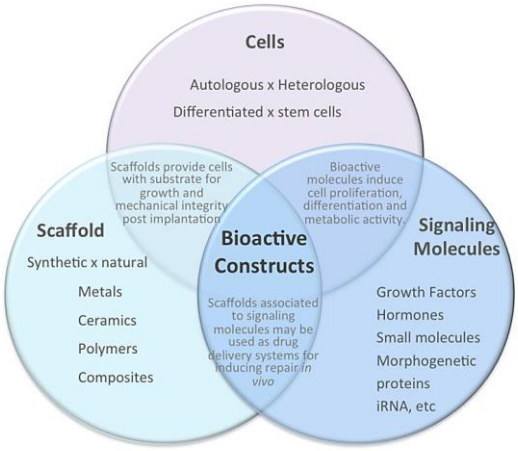


Figure 9. *The three basic components of tissue engineering*

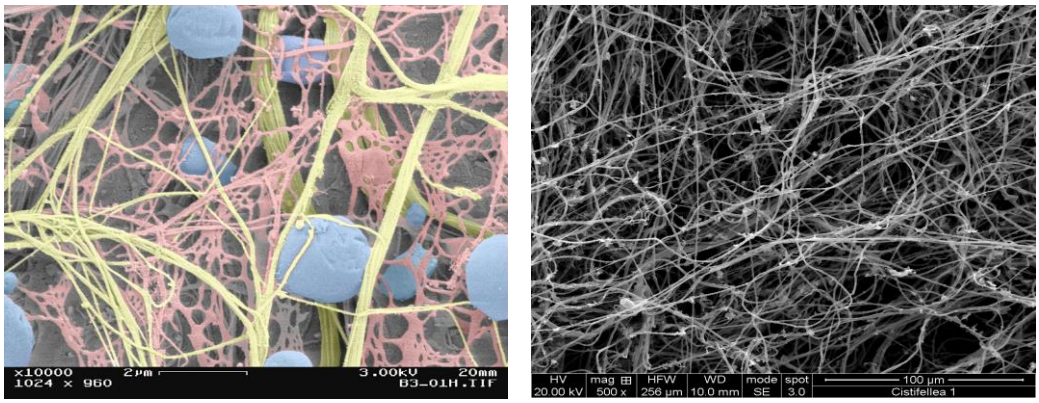


Figure 10. *Comparison of the extracellular matrix and the scaffold implanted on the gallbladder*

Table 1. *Biomaterials and their applications*[56]

POLYMER	ACRONYM	MAIN APPLICATIONS
Polyglycolic acid	**PGA**	Biodegradable sutures, plates and intramedullary rods, degradable fracture plates
Polylactic acid	**PLA**	Intramedullary plates and rods, artificial ligaments, degradable fracture plates, controlled administration of drugs
Styrene	**BS**	Disposable articles, packaging
Butadiene copolymers	**SAN**	Aspirators for blood, haemodialysis machine components
Polyacrylonitrile	**PAN**	Membranes for haemodialysis
Polyamides		SUTURES
Polycarbonate	**PC**	Membranes for oxygenators, and haemodialysis, blood lines
Polyhydroxymethacrylate	**PHEMA**	Contact lenses, artificial ligaments
Polyethylene (PM > 2000000)	**UHMWPE**	Joint surfaces, orthopaedic plates, femoral stem coatings
Polyethylene terephthalate	**PET**	Vascular prostheses, suture rings, transcutaneous passages, components of prosthetic valves, components for cardiac assistance
Poly(methyl) methacrylate	**PMMA**	Bone cement, contact lenses, intraocular lenses, membranes for haemodialysis, dentistry materials
Polypropylene	**PP**	Syringes, membranes for oxygenators, suture threads
Polytetrafluoroethylene	**PTFE**	Vascular prostheses, components of prosthetic valves, artificial ligaments, coatings
Polyurethanes	**PURs**	Catheters, tracheal tubes, prosthetic valves, ventricular sacs, haemocompatible coatings, controlled administration of drugs
Polyvinyl chloride	**PVC**	Blood bags, disposable gloves, endotracheal tubes, catheters, artificial skin, implants for plastic surgery, vascular prostheses, catheters
Silicon		Artificial skin, implants for plastic surgery, vascular prostheses, catheters

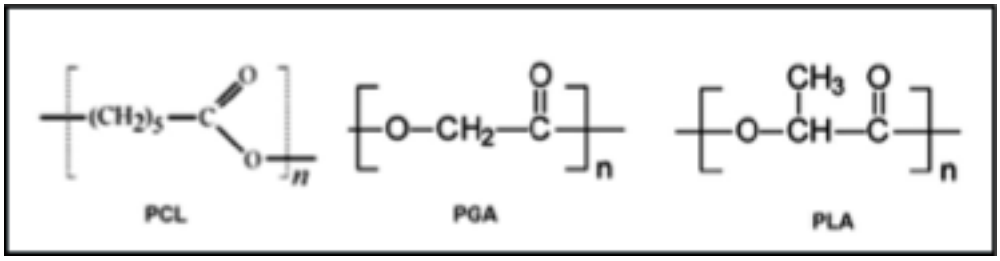


Figure 11. *Repetitive units of the synthetic polymers PCL, PGA and PLA*

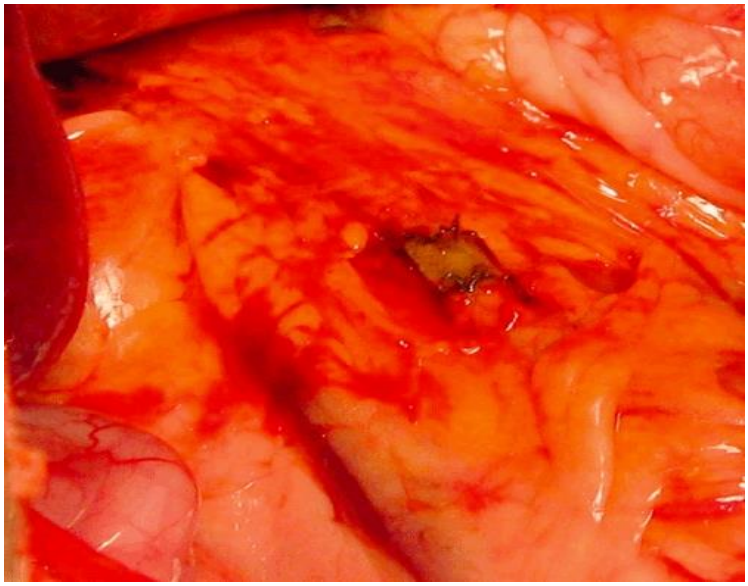


Figure 12. *Tubular grafts on common bile duct in a canine model*.[69]

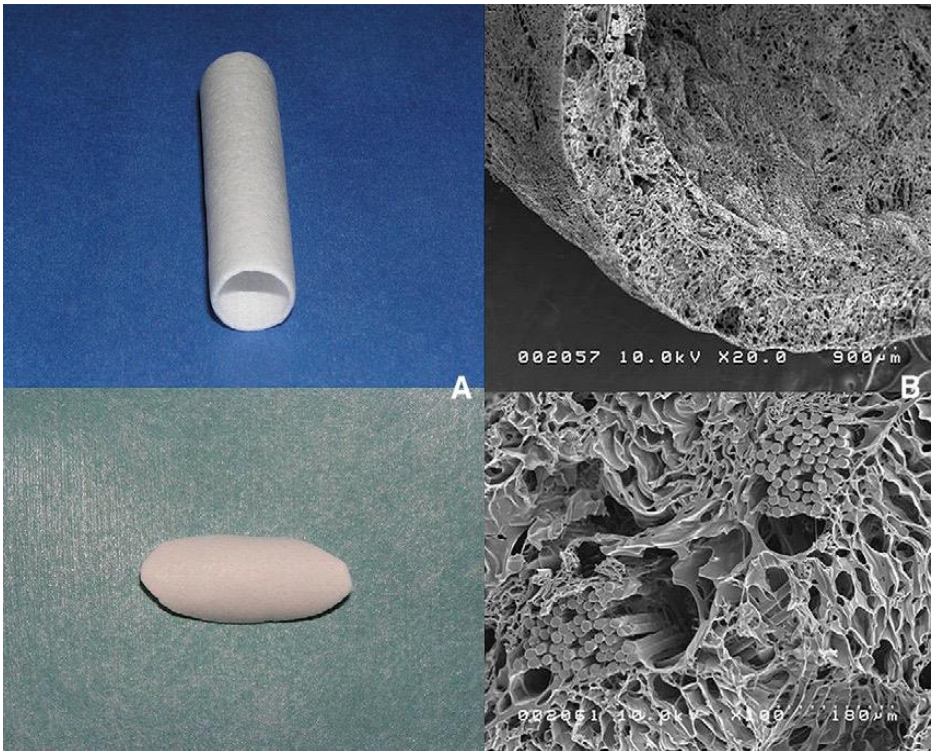


Figure 13. *Biopolymer (BAP tube) under macroscopy* (**A**) *and electron microscope* (**B**)[68]

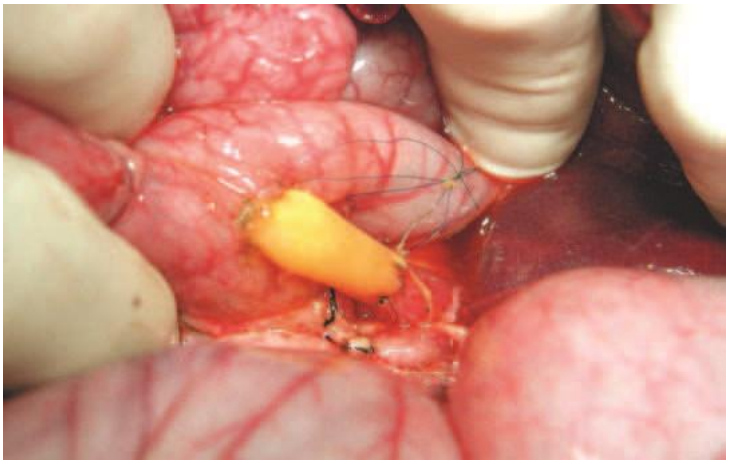


Figure 14. *Bilio-duodenal anastomosis with cellularised scaffold ("Bile duct organoid units")*[70]

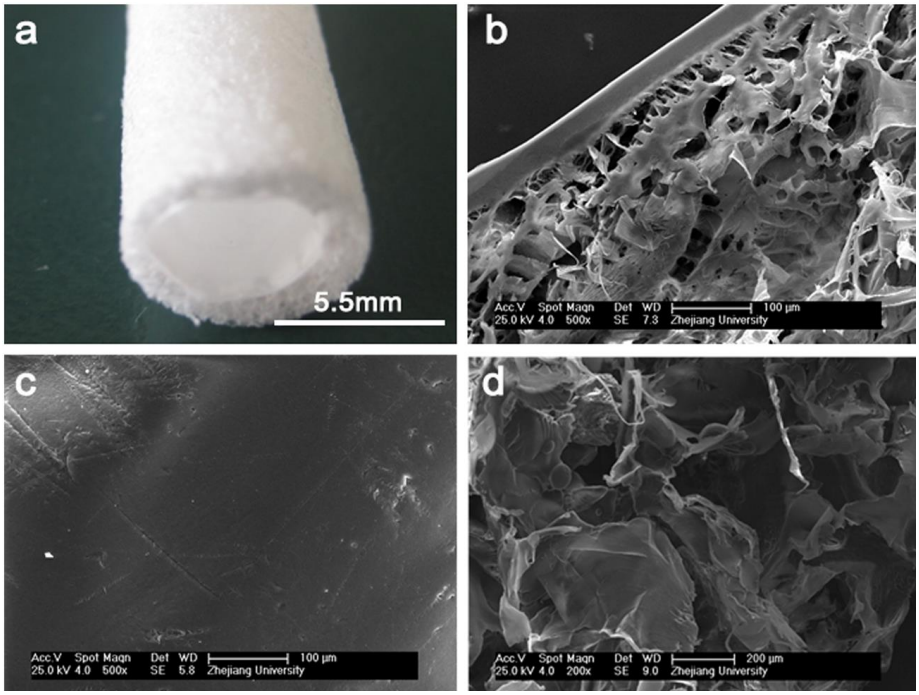


Figure 15. *Biopolymer (BAP tube) under macroscopy* (**A**) *and electron microscope* (**B**)[68]

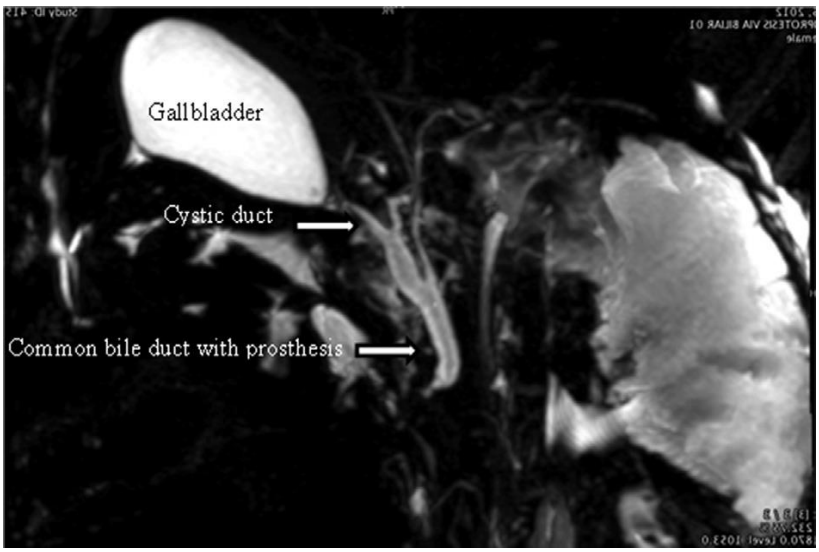


Figure 16. *The magnetic resonance cholangiography at six months shows the absence of stenosis and/or fistulas*[72]

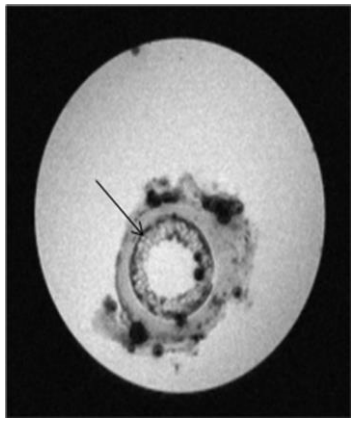


Figure 17. *The magnetic resonance cholangiography shows intraluminal reepithelialisation and the formation of a bile neo-duct around to the scaffold*

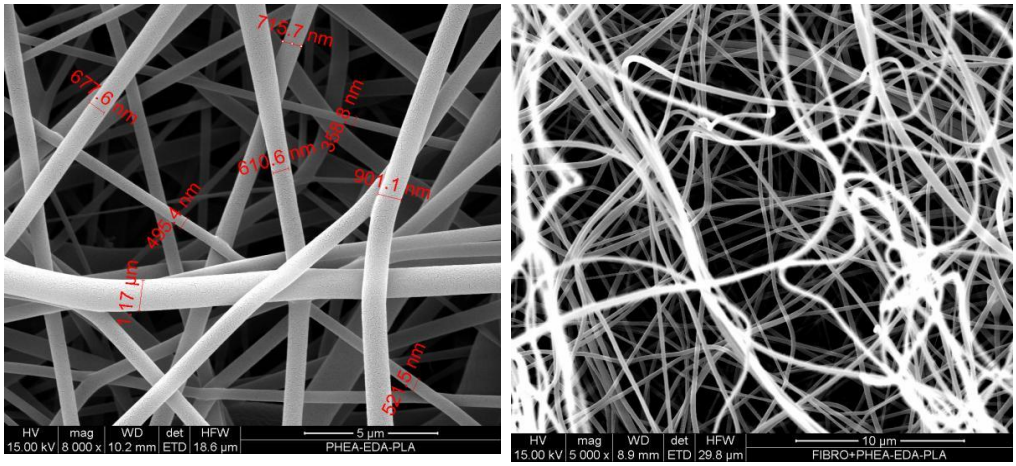


Figure 18. *Scanning electron microscopy (SEM) of PHEA-PLA+PCL fibres*

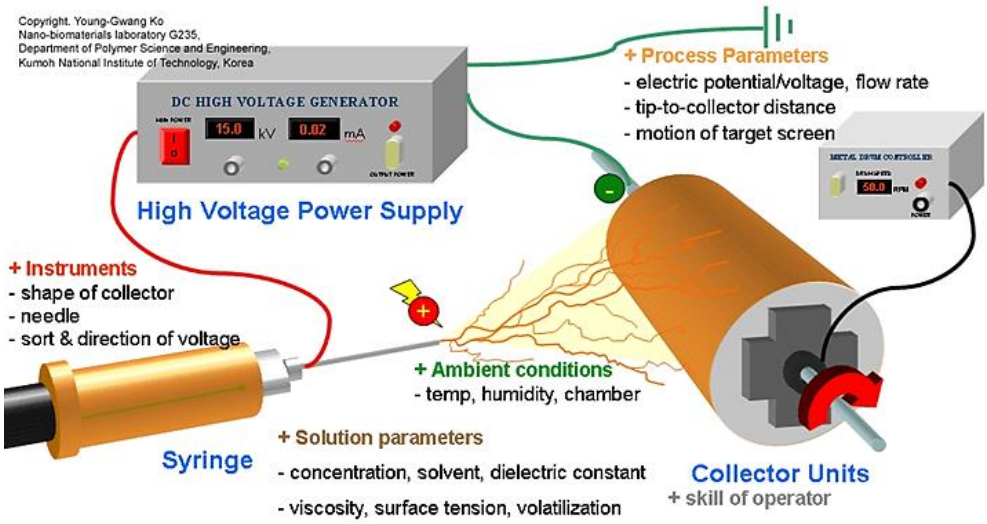


Figure 19. *Electrospinning diagram*[75]

Figure 20. *Tubular scaffold in PHEA-PLA+PCL*

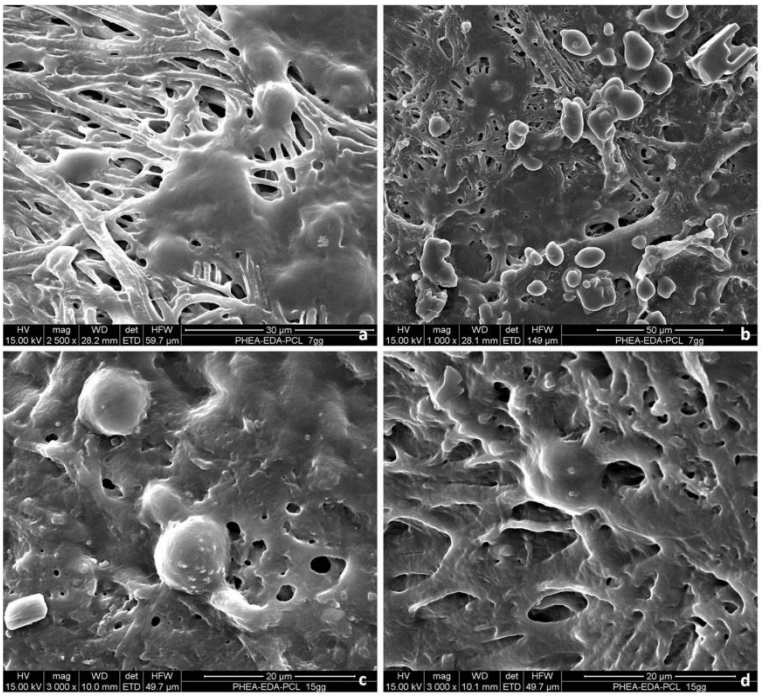


Figure 21. *PHEA-PLA-PCL 7 days (**a, b**) and 15 days (**c,d**) after implantation*.[74]

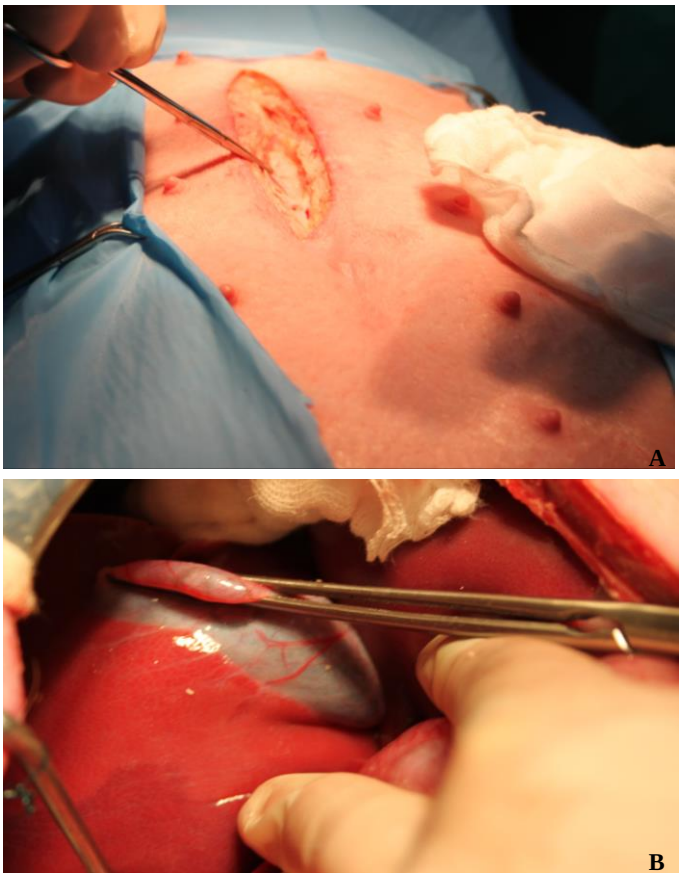
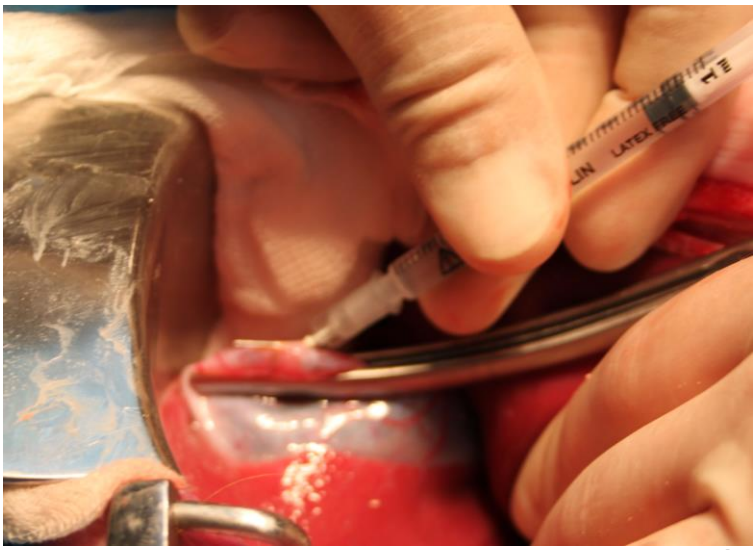
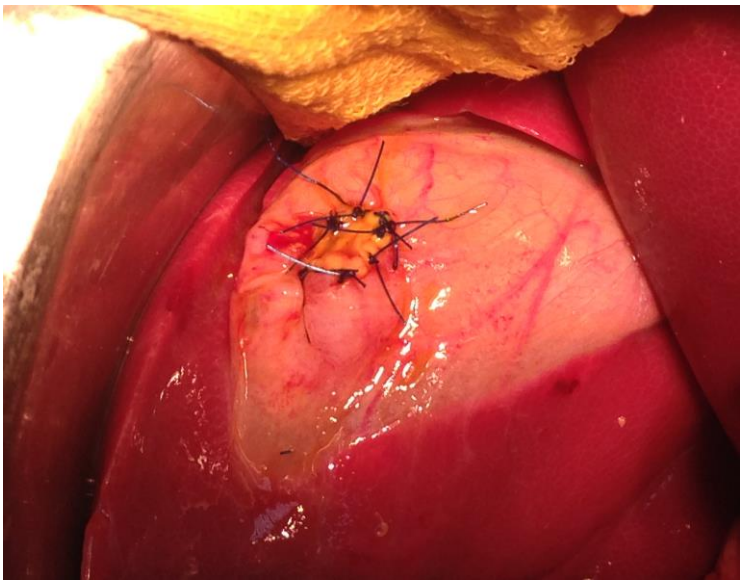


Figure 22. *Median longitudinal incision (**A**) and gallbladder isolation (**B**)*



A



B

Figure 23. *Incision of the gallbladder fundus (**A**) and suture of scaffold with interrupted stitches (**B**)*

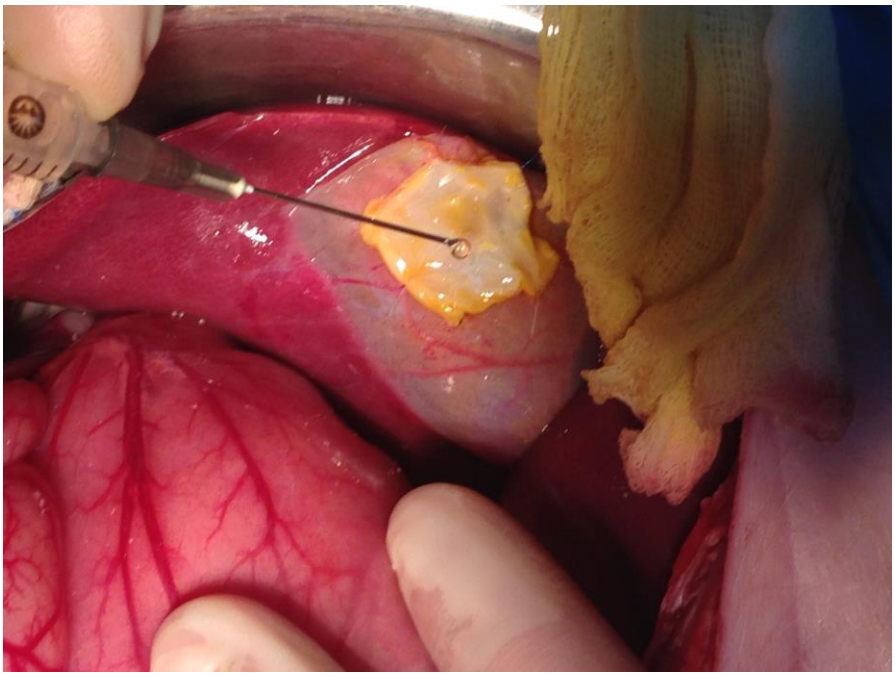
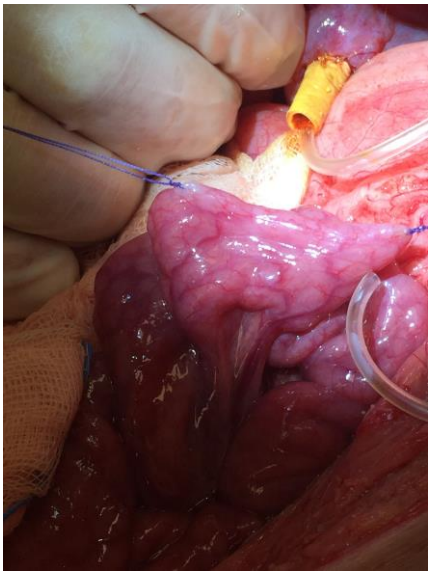
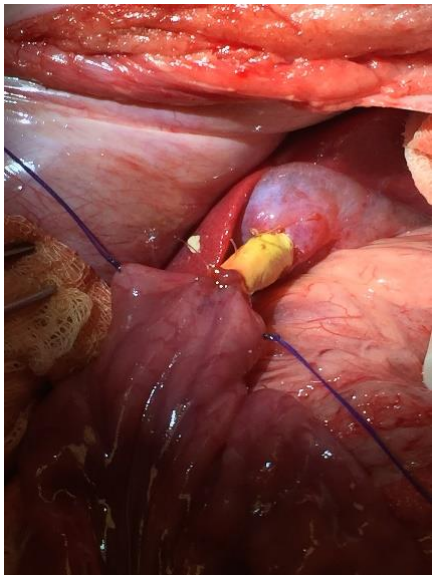


Figure 24. *Application of platelet gel and supernatant present in the test tube*





Figures 25-26. *Scaffold-gallbladder anastomosis with interposition of plastic stent*

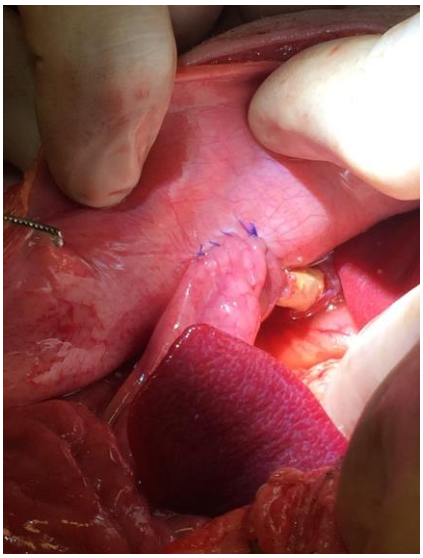
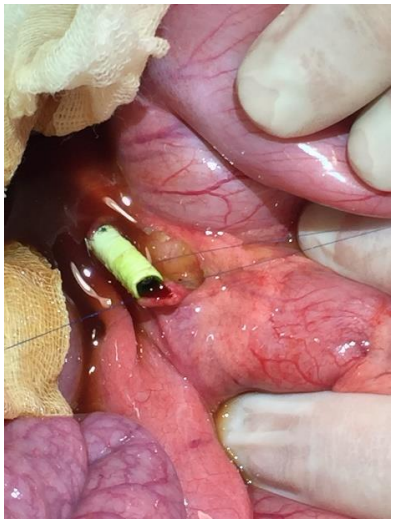
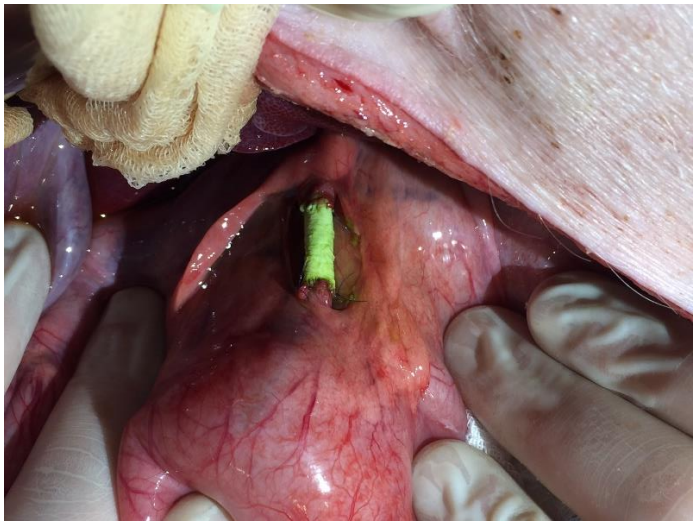


Figure 27. *Jejunal loop anchored to the abdominal wall via continuous suture.*

Figure 28. *Schematic representation of the surgical procedure.*

Figures 29-30. *Termino-terminal anastomosis between the scaffold and CBD.*

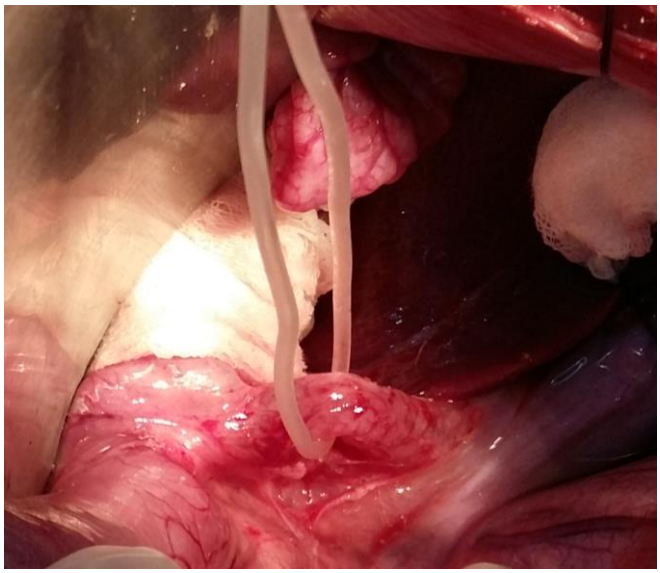


Figure 31. *Duct-CBD isolation.*

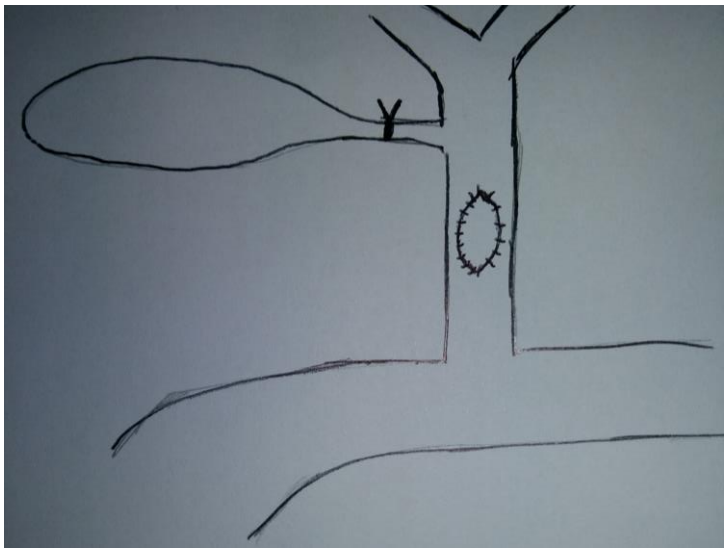


Figure 32. *Schematic representation of procedure.*

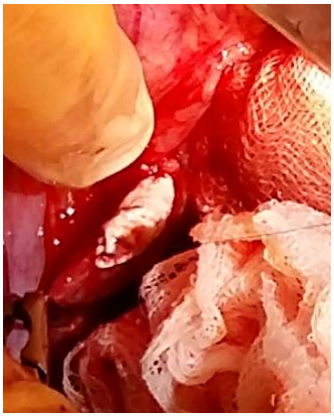


Figure 33. *Scaffold sutured on CBD.*

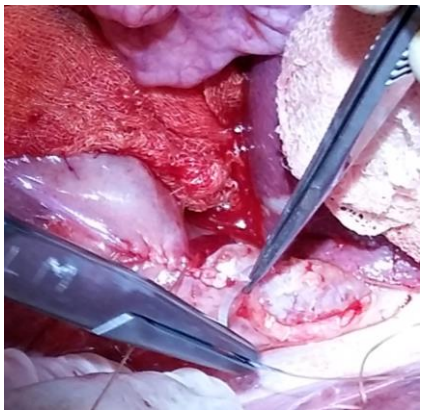


Figure 34. *Closing of visceral peritoneum on CBD.*

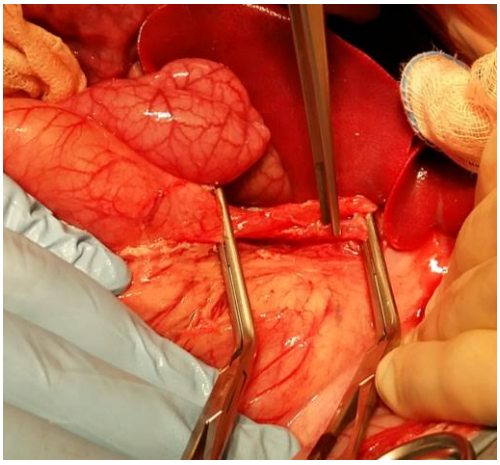
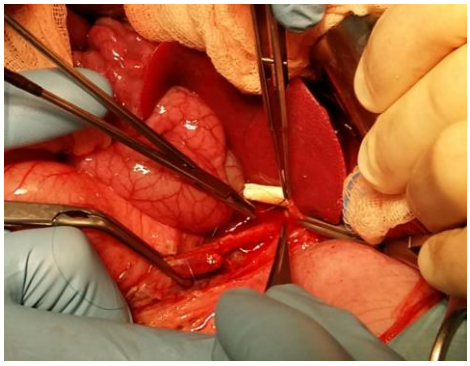
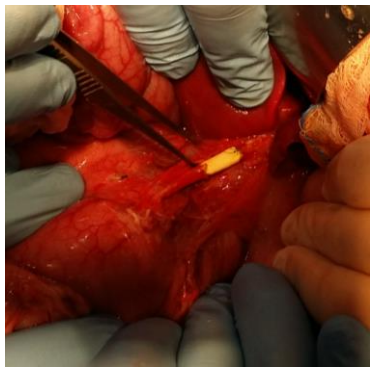


Figure 35. *Anatomy of bile ducts and CBD found in the porcine model.*
(NB. The clamp shows the left hepatic duct)





Figures 36, 37. *Anastomosis of scaffold on the left hepatic duct.*

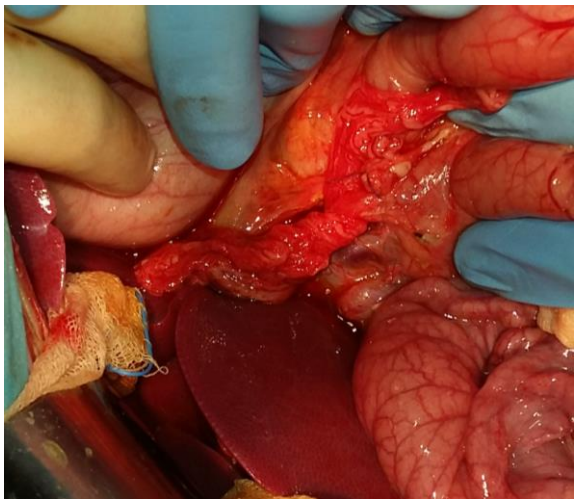


Figure 38. *Small omentum wrapped spirally around the left hepatic duct.*

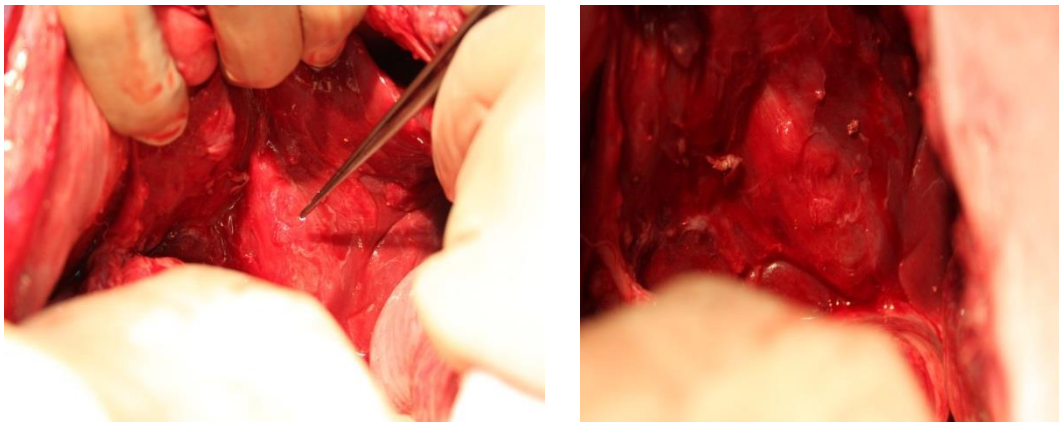


Figure 39. *Exploratory laparotomy of the scaffold*

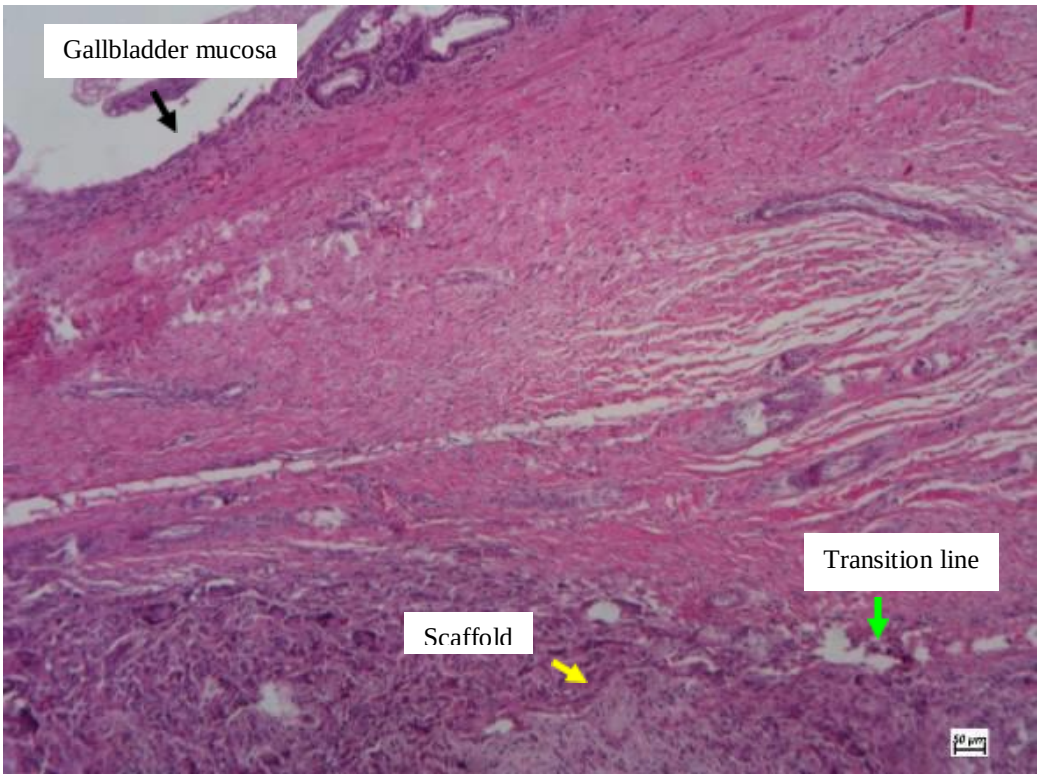


Figure 40. *Transition point between the scaffold and gallbladder*

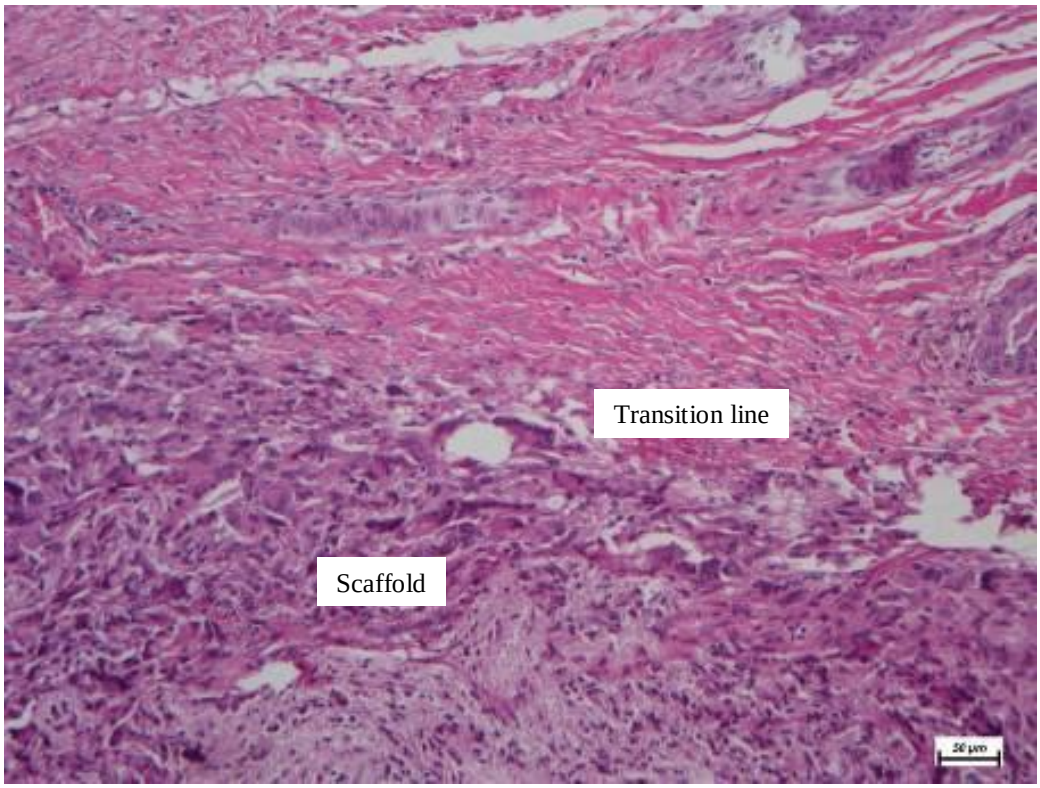


Figure 41. *Transition point between the scaffold and gallbladder EE x10*

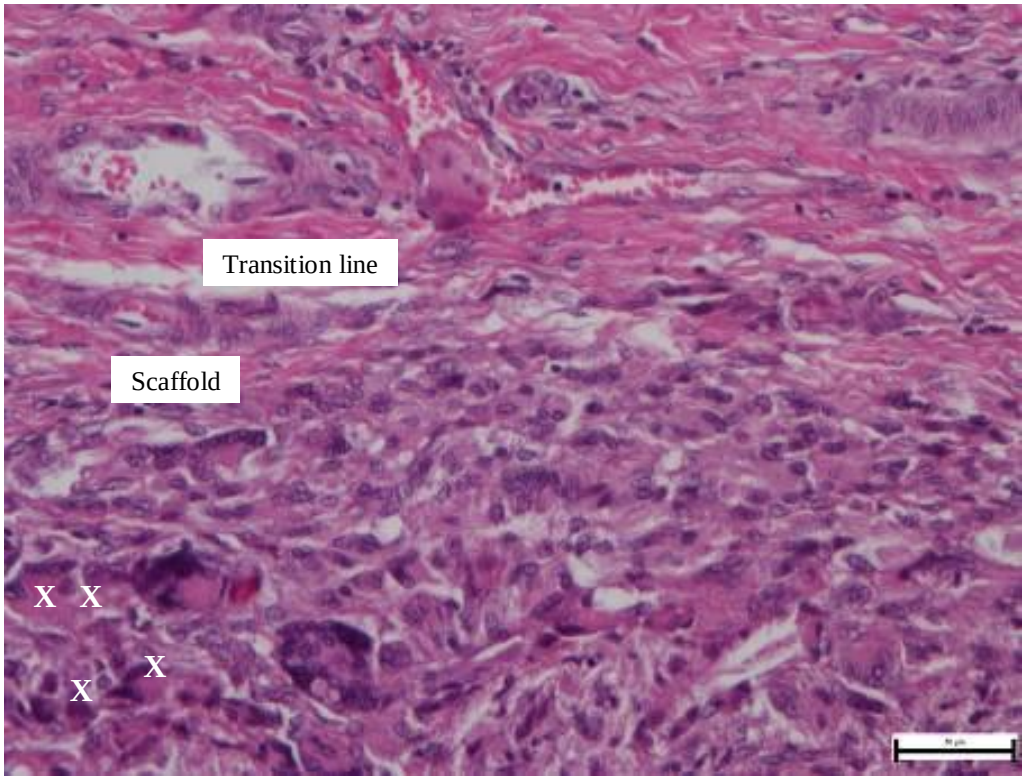


Figure 42. *Presence of inflammatory cells consisting of lymphocytes, macrophages and giant cells (indicated with an x). EE x20*

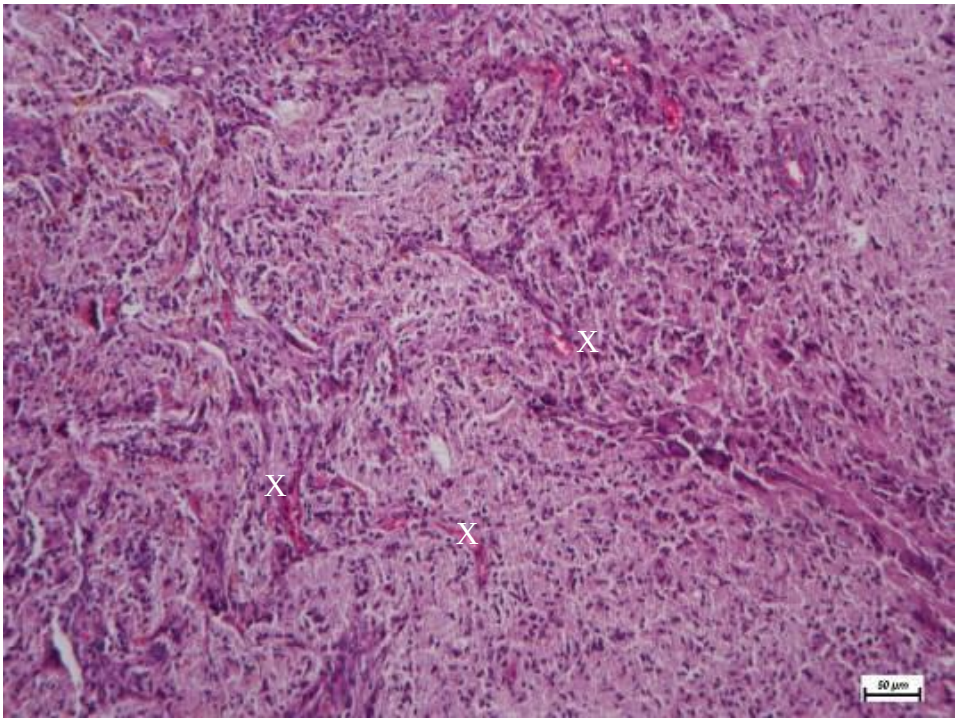
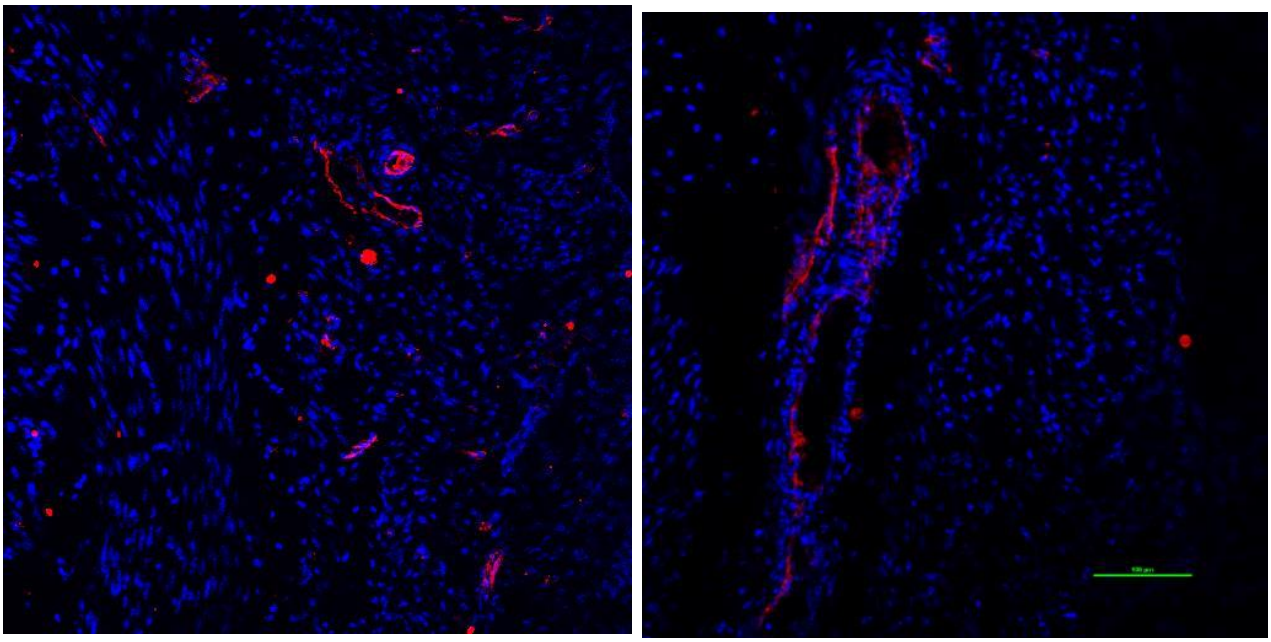
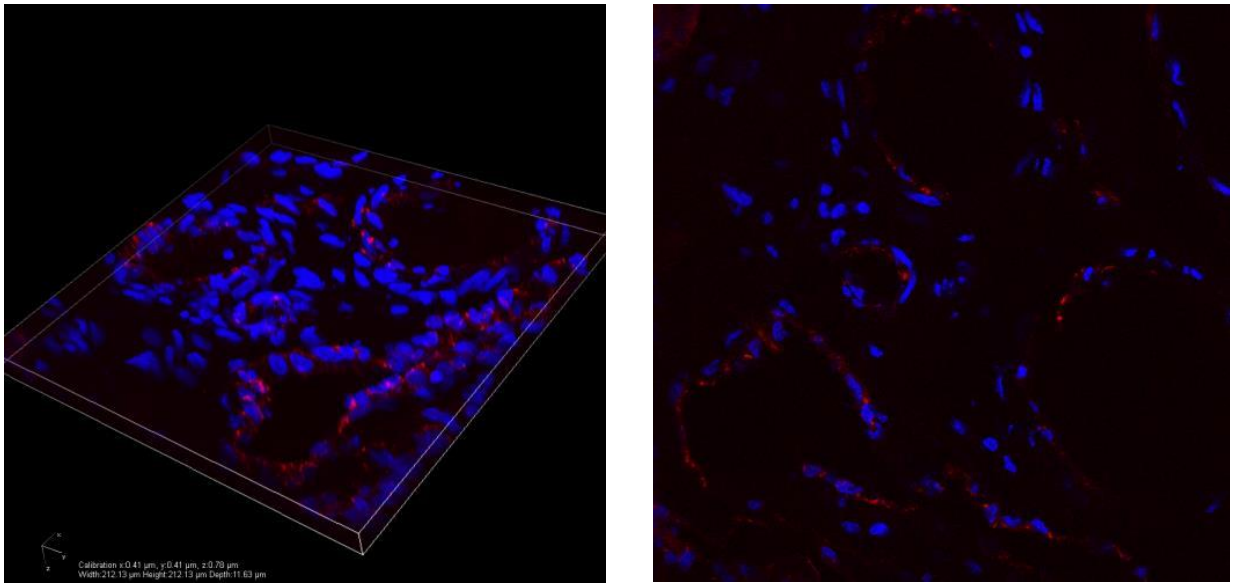


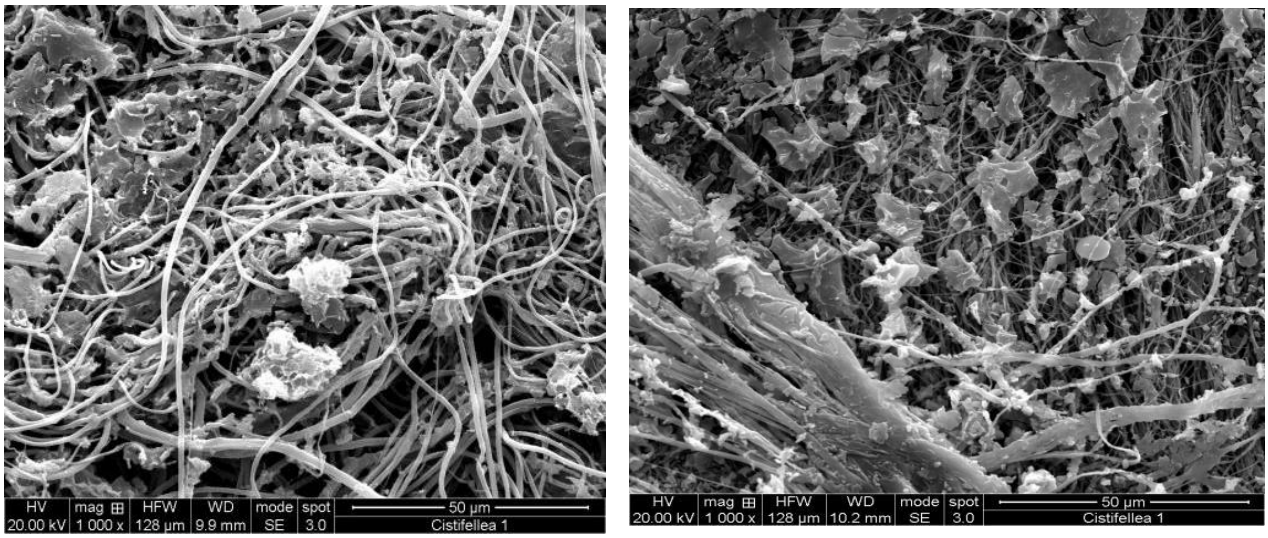
Figure 43. *The scaffold is incorporated with fibrous tissue forming several vessels(x). EE X10*



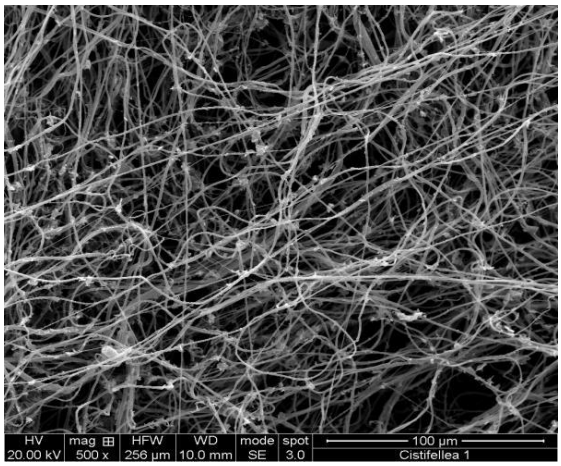
Figures 44-45. Analysis under confocal microscopy (Nikon a1), 40x magnification. Nuclei (blue), anti-CD31 (red)



Figures 46-47. Analysis under confocal microscopy (Nikon a1), 60x magnification. Nuclei (blue), anti-CD31 (red)



Figures 48-49. *Lymphocytes and macrophages on the SEM scaffold*



Figures 50-51. *Residual scaffold structure*

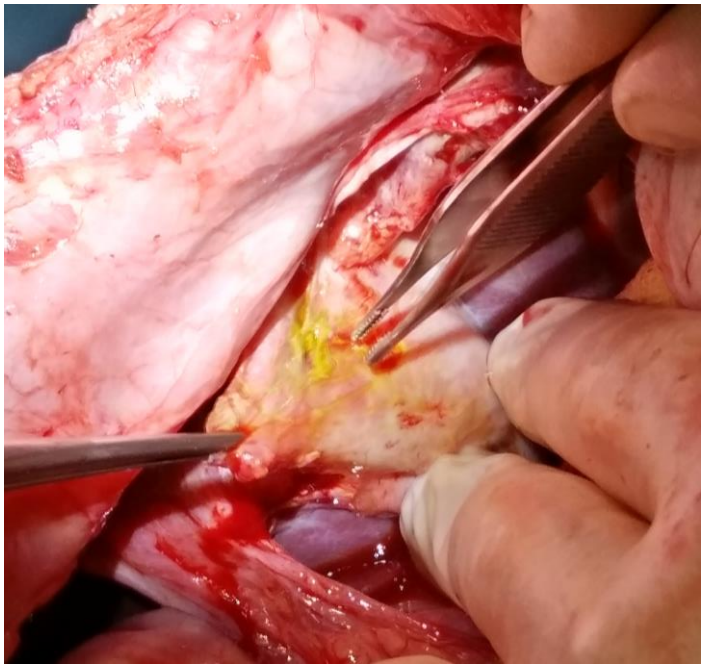
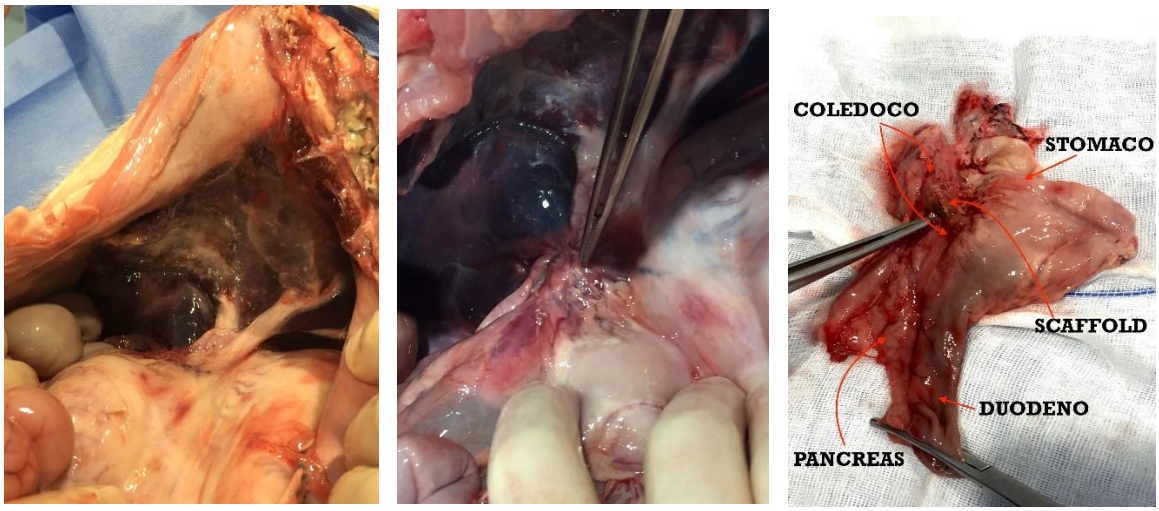


Figure 52. *Obliterated gallbladder stump and healed wound in the gallbladder.*



Figures 53, 54, 55. *Autopsy on porcine model.*

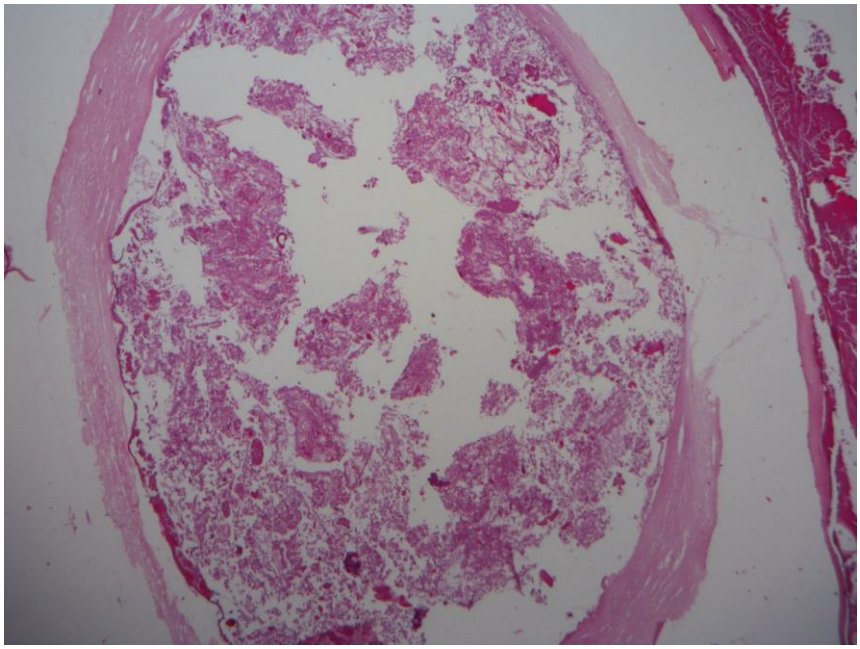


Figure 56. *Common bile duct scaffold*

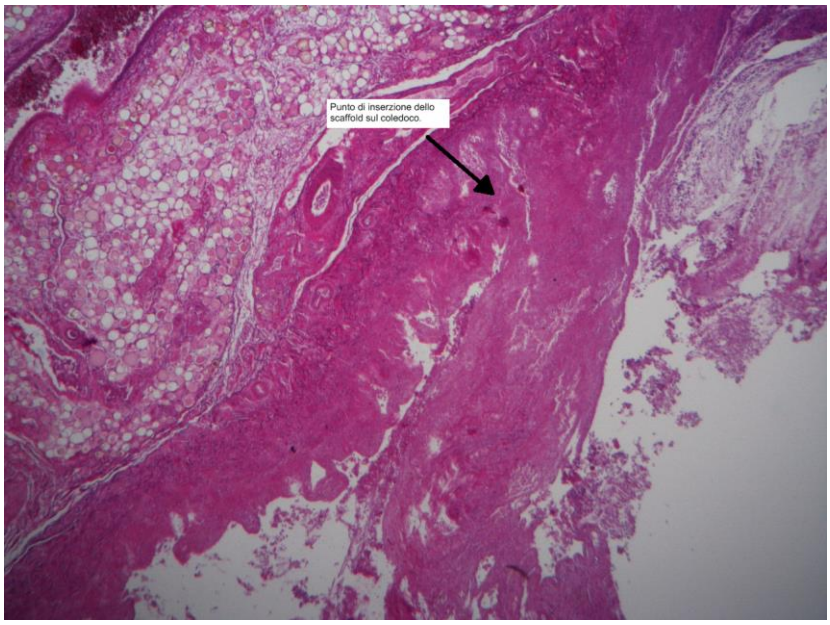


Figure 57. *CBD scaffold: area of integration between CBD and scaffold*

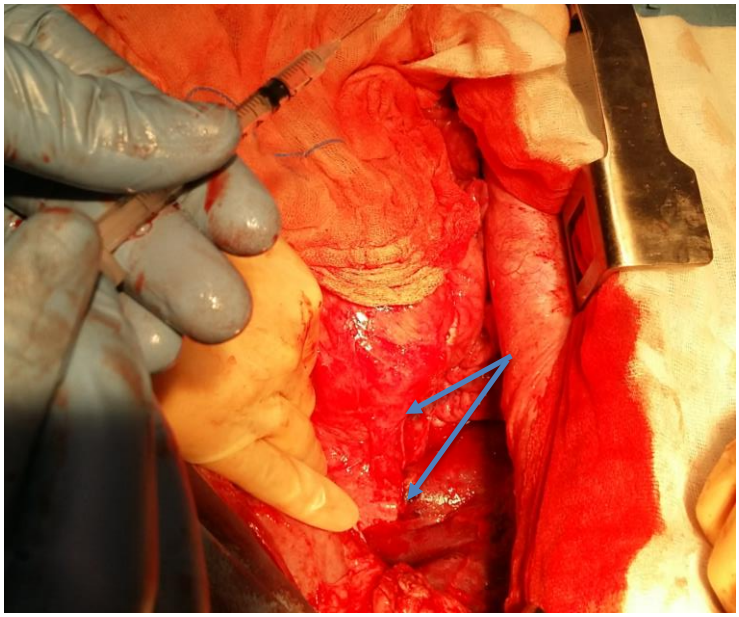


Figure 58. *Reconstructed common bile duct.*

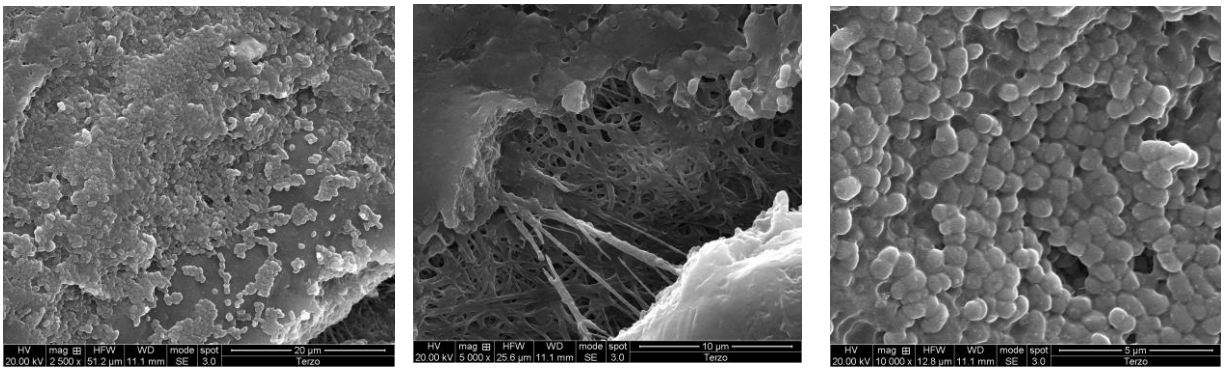


Figure 59. *Scaffold under electron microscopy at different levels of magnification (2500x left, 5000x centre, 10000x right)*

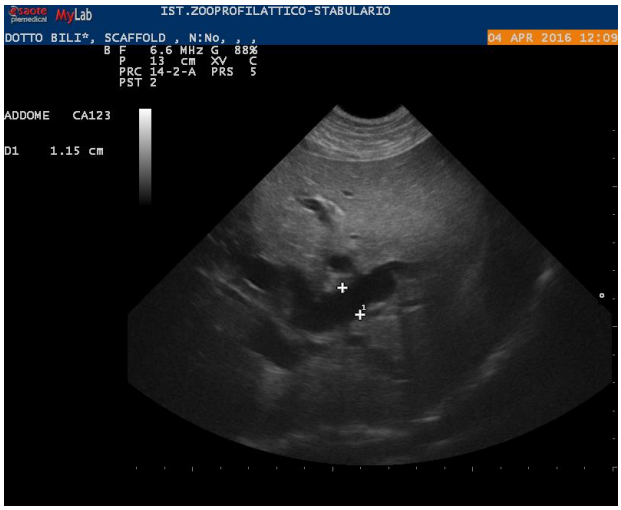


Figure 60. *Abdominal ultrasound of porcine model: dilation of left hepatic duct (DT: 1.5 cm).*

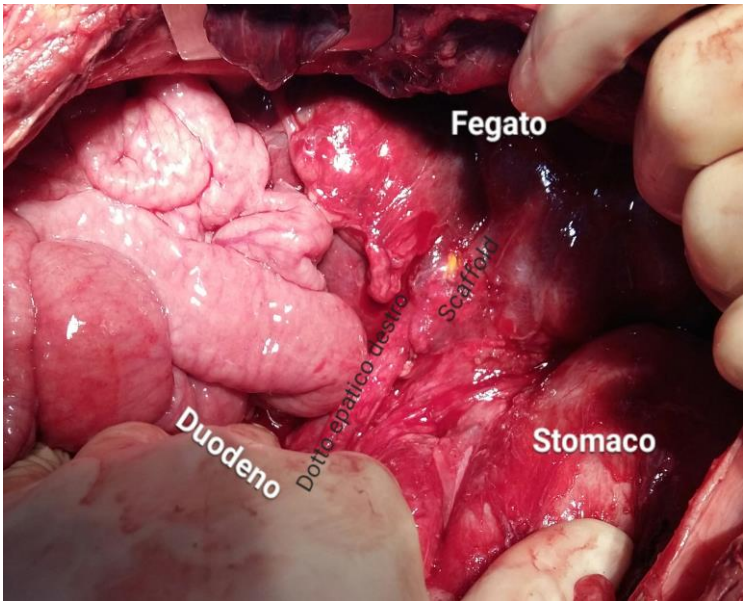


Figure 61. *Explantation of scaffold on the left hepatic duct.*

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