Long-term evaluation of histological alterations following urinary bladder augmentation performed in childhood – Clinical studies and rodent experiment

PhD Thesis

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Contents

I. Introduction

Urinary bladder augmentation is performed according to well-defined indications, mainly due to low bladder capacity caused by congenital organic bladder dysfunction (meningomyelocele, bladder exstrophy). The primary goal of the surgery is to increase bladder capacity, to ensure a low-pressure system in order to protect the long-term kidney function. Bladder augmentation is only indicated when all conservative and minimal invasive treatment options have failed. Together with the storage functions, holding urine and emptying of the bladder have to be treated as well. In case of augmentation cystoplasty, if necessary, urinary continence can be reached with an additional bladder neck procedure. Urine emptying is usually achieved by clean intermittent (self) catheterization (CIC) via the native urethra or through an artificial continent channel (e.g. vesico-appendico-cutaneostomy).

Several surgical techniques are known for bladder augmentation, however, using a segment of the gastrointestinal (GI) tract is still the preferred one. The main procedures are gastrocystoplasty (GCP), when the stomach, ileocystoplasty (ICP), when the ileum, and colocystoplasty (CCP), when the large bowel is used for urinary bladder augmentation. Nowadays ICP is the most commonly performed method.

Integration of enteral mucosa into the urinary system leads to numerous complications. At least 30% of them develop silently, thus long-term follow up of the patients is mandatory. The most serious complication is the malignant transformation of the augmented bladder.

Bladder augmentation gained favor in the ‘80s and ‘90s. After mid- and long-term follow-up of the patients, several case reports were published about malignant tumors which developed in the augmented bladder.

Prevalence of tumors developing in the augmented bladder is estimated to cca. 4-5%. Cancers developed mainly in patients with spina bifida, bladder exstrophy and posterior urethral valve. According to recent data, tumor formation can be expected after a minimum of 10 years following augmentation cystoplasty. Malignancies developing in the augmented bladder usually have high malignant potential and recognized late with metastasis. Therefore, its mortality is high. The type of tumors are usually adenocarcinoma and transitional cell carcinoma, mostly developing in the anastomosis line between the bladder and GI segment.

Several factors, like intestinal bacteria in the urine, increased cell-turnover due to chronic inflammation, or the pathologic interaction between the different epithelia anastomosed together are presumed as the cause of histological alteration. Further risk factors are thought to be the following: immunosuppression, gastrocystoplasty, the cause of the bladder dysfunction itself (MMC, bladder

exstrophy), and general environmental factors related to tumor genesis (e.g. smoking, alcohol consumption). Urinary tract infections and bladder calculi are still investigated as promoters, but no evidence supports their carcinogenic effect.

There is no widely accepted protocol on how to follow the augmented patients. Various surveillance protocols exist for the early diagnosis of tumor formation, including cytology, imaging studies, cystoscopy with or without biopsy sampling performed annually, biannually, or by other conventions.

In the last ten years, some groups published about the low effectivity of surveillance cystoscopy, which advocated the review of the current protocols of different centers. Husmann et al published the most comprehensive clinical data related to surveillance cystoscopies. This study suggested a protocol to perform the endoscopies only in symptomatic cases to detect the tumor formation in time.

The surgical unit of the Department of Paediatrics in Pécs is the national center for congenital urinary bladder dysfunctions (MMC, bladder exstrophy). Bladder augmentation in childhood is performed only at this center, and the follow-up of the patients is done by the same team as well. Investigations include the anatomical and functional assessments of the urinary tract, urine sampling, searching for bladder stones and metabolic status check-up. From the fourth postoperative year, surveillance cystoscopies together with biopsy sampling are performed. Patients are followed prospectively to gain better knowledge about the short-, mid- and long-term complications following bladder augmentation cystoplasties.

In 2002, our group published a series involving 20 colocystoplasty (CCP) and 15 gastrocystoplasty (GCP) patients. Histological results of the biopsies, frequency of positive urine cultures and bladder stone occurrence were analyzed and correlated to each other. Sporadic mucosal alterations were found as squamous metaplasia, other metaplasia and, in one case, dysplastic lesion. A significant correlation between the nature of histological changes and the frequency of bacterial colonization after CCP was found. Bladder stones have not correlated with the histological alterations in either group. Biannual screening for histological alterations beginning after 10 years following augmentation cystoplasty was recommended.

Many factors can make tumor research in humans related to bladder augmentation difficult. Rat models became popular in the last 3 decades to investigate tumor genesis in detail, among others. In rat model, complications can be examined early, due to their relatively short, 2-3 years life-expectancy, in which there is a high possibility for tumor development. From the ‘90s many articles were published about performing bladder augmentation in rats, mainly ileocystoplasty. Beside the tumors, some groups observed urothelial appearance on the normal mucosa of the intestinal segment used for augmentation, similar to the urothelization of seromuscular

enterocystoplasties. The pattern of this urothelization and the possible underlying role has not yet been investigated.

II. Aims of the study

A.) To re-evaluate the efficiency of the follow-up protocol used at the University of Pécs, Department of Paediatrics, Surgical Unit, aimed to reveal mucosal alterations following augmentation cystoplasties and their relation to bacterial colonisation and bladder stone occurrence.

The following questions were investigated:

1.) Over sixteen years (between 2002 and 2018), has any malignancy developed following CCP or GCP in the same patients who had participated in the previous study?

2.) Does the type of gastrointestinal tissue used for augmentation or the underlying disease (MMC and bladder exstrophy) correlate with the histological changes over these 16 years (between 2002 and 2018)?

3.) Is there any significant change over the period of 16 years in the frequency of positive urine cultures or in the stone occurrence?

4.) Is there any difference in the incidence of positive urine cultures and stone occurrence between CCP and GCP or MMC and bladder exstrophy?

5.) Are the results of urine cultures significantly influencing the histological findings?

6.) Is surveillance cystoscopy with random biopsy sampling a feasible method to detect and follow the histological alterations and predict tumor formation?

B.) Parallel with the clinical study, based on the literature, the authors adopted a rodent model, in order to investigate the long-term tumor genesis and the pattern of urothelization following augmentation cystoplasty with ileal segment.

III. Materials and methods

III.1. Clinical investigation

To re-evaluate our current follow-up protocol, the 35 original patients were included in the current study, who were evaluated previously in 2002. In 20 patients colocystoplasty (CCP), in 15 patients gastrocystoplasty was performed. According to the protocol published in the original article, initial biopsies were taken at the time of augmentation cystoplasty. Following the operation, repeated cystoscopies were performed, and biopsies were taken biannually from the 4th postoperative year. Two samples for histology were harvested from the mucosa of the native urinary bladder, from the colonic- or gastric-segment used for augmentation, and from the anastomosis line. The mucosal biopsies were taken endoscopically, or occasionally via open surgery (in case of bladder stone removal). In both groups, representative sections were examined with routine hematoxylin and eosin (HE) staining and evaluated by pathologists. Permission for cystoscopic examinations and biopsies was obtained from all individuals. Consents for the clinical research and the processing method of the data were accepted by the Regional Research Ethics Committee (3043/2007).

Patients who did not show up on any of the control examinations were questioned about tumor development over the phone, but they were not included in the study.

As in the original study, the frequency of positive urine cultures representing significant ($>10^5$ colony-forming units (cfu)/ml) bacterial colonization and stone formation were also regularly recorded.

Beside the descriptive analysis of the data, the following statistical tests were used, listed in the same order as the aims of the study:

1.) Over sixteen years (between 2002 and 2018), has any malignancy developed following CCP or GCP in the same patients who had participated in the previous study? ; 2.) Does the type of gastrointestinal tissue used for augmentation or the underlying disease (MMC and bladder exstrophy) correlate with the histological changes over these 16 years (between 2002 and 2018)?

Related to the nature of the histological changes, the comparison between the CCP and GCP groups was done by Chi-squared test. Due to the nearly equal number of patients with meningomyelocele (MMC) and bladder exstrophy (BE) in the colocystoplasty (CCP) group, comparison between the two underlying conditions was done with the same statistical method.

3.) Is there any significant change over the period of 16 years in the frequency of positive urine cultures or in the stone occurrence?

Within the CCP and GCP groups, differences between the incidence of positive urine cultures in the previous evaluations and the evaluations done during this study were analyzed with Wilcoxon signed-rank test. In terms of stone occurrence, due to the nature of the data, only descriptive analysis was made.

4.) Is there any difference in the incidence of positive urine cultures and stone occurrence between CCP and GCP or MMC and bladder exstrophy?

The difference between the frequency of positive urine cultures in the two groups (CCP vs GCP) and etiologic factors (MMC vs BE) was analyzed with Mann–Whitney U test. The difference between the stone occurrence was depicted descriptively only.

5.) Are the results of urine cultures significantly influencing the histological findings?

Association between the histological findings and the results of the urine cultures within groups were analyzed by Chi-squared test.

For statistical calculations the IBM software, Statistical Package for the Social Sciences (SPSS) Statistics version 25, has been used ($p<0.05$ considered to be statistically significant).

III.2. Animal model

The animal studies were carried out at the Animal Facility of the University of Pécs, Medical School. Permission for animal studies was obtained (BA02/2000-15/2012).

The investigation was done on 45, 3-5 months old, 200-400 grams, male Wistar rats. In 30 rats ileocystoplasty (ICP), in 15 rats sham operation was performed.

To empty the gastrointestinal tract, all animals were starved for 1 day prior the surgery. Anaesthesia was achieved with diazepam (Seduxen, dose: 10 mg/kg) and ketamine (Calypsol, dose: 50 mg/kg). Antibiotic prophylaxis was applied in all animals and administered intramuscularly, for 3 day perioperatively (cefuroxime - Zinacef, dose: 50 mg/kg).

For precise manipulation, fine and microsurgical instruments as well as 3,5x loupe magnifications were used.

III.2.1. Ileocystoplasty (ICP) – 30 rats

After the preparation of the surgical site (abdomen), median laparotomy was done. The urinary bladder was dissected and stay sutures were placed into it. An approximately 1,5 cm long ileal segment with a long vascular pedicle was chosen for augmentation. After the sceletization of the ileal segment, it was cut out from the GI tract and folded to the bladder. Continuity of the small bowel was constructed with an end-to-end anastomosis between the free bowel ends, using 6/0 braided absorbable suture. The ileal segment was detubularized and the mucosa was irrigated with physiologic saline solution. The detubularized ileal segment was then sutured to the longitudinally incised urinary bladder with one layer running suture, using 6/0 braided absorbable suture.

To prevent hypothermia and dryness of the organs, warm physiologic saline solution was irrigated during surgery. After the augmentation, the abdominal cavity was washed out with saline solution and closed in two layers, using 3/0 braided running sutures.

Postoperatively, the animals were only water for 24 hours. As postoperative analgesia, ketoprofen was used.

Animals were euthanized after 12 (Group A), 18 (Group B) and 24 (Group C) months postoperatively.

III.2.2. Sham-operation – 15 rats

In this group only sagittal cystotomy and closure of the urinary bladder was performed with 6/0 braided running suture. Everything else was done exactly the same way as in ICP groups. Every animal in this group was euthanized 24 months after the procedure (Group D).

III.2.3. Harvest of the samples and histological studies

Until termination the animals were kept in the Animal Facility of the University of Pécs, Medical School.

Tissues were harvested under the same general anaesthesia which was used for the operations. Through median laparotomy the augmented bladder with ileal segment was dissected. First the bladder neck, then the ureters, at last the vascular pedicle of

the bowel segment used for augmentation was cut and the augmented bladder was removed.

The bladder was sliced in a mid-sagittal plane. At first the mucosa of the bladder, the ileum and the anastomosis line were examined macroscopically for any mucosal alterations under loupe magnification. Samples were then placed in 6% formaldehyde, in a slightly stretched position. Orientation during the process was important, to allow the examination of both the bladder, the ileal patch and the anastomotic line on the same preparation.

After harvesting the augmented bladder, animals were euthanized using intracardiac potassium-chloride. Processing the sham-operated animals was done similarly as described in case of ICP.

The fixed preparations were embedded in paraffin and 5 µm sections were made perpendicular to the anastomosis line. Routine HE staining was used. An independent pathologist assisted the evaluation of the sections.

IV. Results

IV.1. Clinical investigation

In summary, 19 patients from the CCP group and 11 patients from the GCP group were eligible for the follow-up until 2018. Five patients (CCP – 1, GCP – 4) were excluded from the study, as they did not want to participate in any of the control examinations. According to their answers given over the phone, none of them were diagnosed with cancer during the period of the study (between 2002 and 2018).

In the CCP group, 10 patients had BE and 9 had MMC. The GCP group consisted of patients with MMC. Mean patient age at operation was 11.8 (range 6–21) years. Average elapsed time between evaluations was 11 years in both groups. The mean follow-up period of re-evaluation after CCP and GCP was 19.6 (range 13–27) and 15 (range 6–20) years.

Results are detailed in the order of the questions raised in the aims section:

1.) Over sixteen years (between 2002 and 2018), has any malignancy developed following CCP or GCP in the same patients who had participated in the previous study?

Regarding cystoscopies, no suspicious macroscopic alteration was found. The biopsies have not shown any malignant or premalignant changes either. Beside the normal appearance of the mucosa, different degrees of inflammation and metaplastic signs were noted (*Table 1*).

CCP	**Urothelium (n=55)**	**Anastomosis (n=55)**	**Colon mucosa (n=55)**
Normal	33 (60%)	28 (51%)	17 (31%)
Inflammation	19 (34%)	23 (42%)	38 (69%)
Metaplasia	3 (6%)	4 (7%)	0
GCP	**Urothelium (n=36)**	**Anastomosis (n=36)**	**Gastric mucosa (n=36)**
Normal	10 (28%)	15 (42%)	27 (75%)
Inflammation	20 (55%)	16 (44%)	9 (25%)
Metaplasia	6 (17%)	5 (14%)	0

Table 1: Results of biopsies taken during surveillance cystoscopies after CCP and GCP

CCP group, n=19: One patient was lost to follow-up. No macroscopic lesions were found. Normal mucosa was present in 48% of the samples. One patient did not have any changes over the follow-up period, and four patients had completely normal colonic and urothelial mucosa. One of these patients had metaplastic and dysplastic signs before, while the others showed signs of inflammation previously. Inflammation of the mucosa was found in 48% of the samples as well. The bowel epithelium showed the most significant signs of inflammation. Metaplastic changes in the urothelial cells were found in four patients and in 4% of all samples, and it was seen only in the original bladder and the anastomosis line. Squamous cell metaplasia was found in two cases in the urothelial mucosa and in two cases at the anastomosis line. Two of these appeared intact over time. Colonic metaplasia was found in one case, but the later samples of the same patient did not show this finding again.

GCP group, n=11: Four patients were lost to follow-up. Macroscopically no suspicious growth was noticed. The mucosa did not show any alterations in 48% of the samples. Chronic inflammation was present in 42% in this group, mostly affecting the urothelial mucosa. In contrast, the gastric mucosa showed the least signs of inflammation. Both the urothelial mucosa and the anastomosis showed 11 (10% of all samples) metaplastic changes in four patients. All of them were squamous metaplasia of the urothelium, except for one, which was a colonic metaplasia of the gastric mucosa at the anastomosis line. In two patients the metaplastic alterations did not show up in the subsequent biopsies.

2.) Does the type of gastrointestinal tissue used for augmentation or the underlying disease (MMC and bladder exstrophy) correlate with the histological changes over these 16 years (between 2002 and 2018)?

CCP vs GCP: No cases of malignancy were identified in either group during the follow-up. Data showed significant association between the type of augmentation and the nature of histological changes in the urothelial mucosa ($p = 0.003$) and in the colonic/gastric mucosa ($p < 0.001$). More frequent inflammatory and metaplastic changes were found on the urothelium over these years after GCP. The mucosa of the colon showed more inflammatory changes than the gastric mucosa. (Table 1).

MMC vs BE: Significant association was found between the underlying condition and the nature of histological alterations in the urothelial mucosa ($p = 0.009$) and the anastomosis line ($p = 0.025$). Metaplastic lesions of the urothelium occurred only in patients with bladder exstrophy (BE), who had also undergone gastrocystoplasty for the same condition in childhood. The colonic mucosa did not show any histological changes in this relation.

3.) Is there any significant change over the period of 16 years in the frequency of positive urine cultures or in the stone occurrence?

The rate of bacterial colonization did not change significantly neither in the CCP, nor in the GCP group during the longer follow-up. The re-evaluation revealed more frequent stone formation within the CCP group than the previously published findings (13 vs 30). Out of the 19 patients, 15 had at least one stone, and eight patients had multiple stones. Only one bladder stone was identified in the GCP group, which originated from the kidney.

4.) Is there any difference in the incidence of positive urine cultures and stone occurrence between CCP and GCP or MMC and bladder exstrophy?

None of the patients presented signs of urinary tract infection at the time the samples were taken during this study. The overall mean frequency of positive urine cultures was 64% after CCP, and 31% after GCP. Significant difference was found between the two groups ($p<0.001$). Stones occurred mostly after CCP.

Within the CCP group, the frequency of positive urine cultures did not depend on the underlying disease (MMC vs BE). Presence of stones was more common in BE patients (18 vs 12).

5.) Are the results of urine cultures significantly influencing the histological findings?

The nature of histological changes did not show any relation with the results of the urine cultures in either group ($p>0.05$).

IV.2. Animal model

I.) Ileocystoplasty – Group A, B and C

After 30 ileocystoplasties, 18 (60%) rats survived the planned follow-up period. The mean operation time was 60 minutes (45-90 minutes). The causes of death are detailed in *Figure 1*.

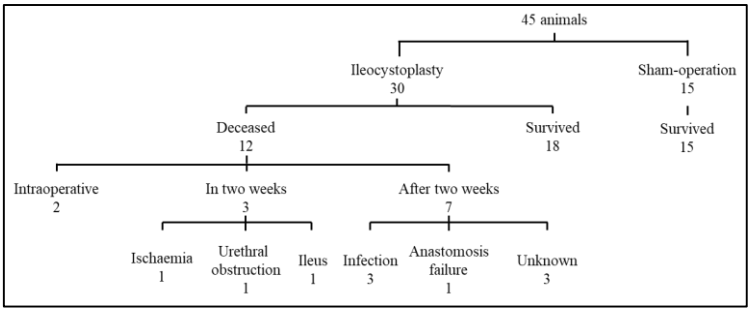

Figure 1: Summary of complications developed after ICP in rats.

In all the 18 surviving animals, mucous bladder content was observed. The ureters and the kidneys were normal. Further macroscopic and microscopic intravesical alterations are discussed in Groups. The summary of these results is presented in *Table 2.*

	Group A 12 months	Group B 18 months	Group C 24 months	Group D Sham
Number of animals	6	6	6	6
Urothelial differentiation of bowel epthelium	+	++	+++	-
Bladder stones	**2**	0	0	0
Squamous cell metaplasia	**1**	0	0	0
Polyp	0	0	**1**	0
Malignancy	0	0	**2**	0

Table 2: Macroscopic and microscopic findings following ICP in rats.

ICP, Group A (12 months)

Bladder stones developed in two animals. One of them presented squamous cell metaplasia on the mucosa of the native bladder. In all of the samples close to the anastomotic line significant urothelial hyperplasia could be seen on the native bladder. On the adjacent villi of the ileal patch, columnar epithelium was replaced by varying amounts of urothelial cells. Cyrpts underlying these heterogenic villi seemed to be shorter, which was more prominent next to the anastomosis as well. Distally from the anastomosis line, the urothelium covered vertically smaller areas, only the tip of the villi. The ultrastructure of the urothelium was normal here.

ICP, Group B (18 months)

No macroscopic alteration was noted except the mucus formation. Microscopically similar, but more significant histological changes were detected, like in Group A. The urothelium replaced more ileal territory horizontally on the ileal patch and vertically along the villi as well. The replacing urothelium showed more hyperplastic signs, like increased nuclear-cytoplasmic ratio, increased euchromatinization of the nuclei, they did not appear to be malignant however.

ICP, Group C (24 months)

In one rat, a polyp-like overgrowth was detected at the anastomosis line, formed by a hyperplastic urothelial epithelium.

In two other rats the mucosa of the ileal patch was significantly thickened. This macroscopic finding was proven to be a transitional cell carcinoma together with an extensive urothelial transformation of the ileal epithelium.

In all of the animals the villi fully disappeared on the ileal side of the augmented bladder and were replaced by hyperplastic urothelium, overlying the remnants of the crypts. Next to the anastomosis line crypts disappeared as well or were replaced by nests of urothelial cells. Only this group showed signs of chronic inflammation.

II.) Sham operation – Group D

All 15 animals survived the planned 24 months follow-up period. In one case a small diverticulum was observed in the anastomosis line. No other macroscopic or microscopic changes were detected.

V. Summary and conclusions

Due to the high complication rate of urinary bladder augmentations, follow-up of the patients is mandatory. From the beginning of the '90s, children operated with this procedure in Hungary are prospectively followed by one team. The most severe and yet less known complication after augmentation is the malignant transformation. As the patients get older, the possibility of tumor formation increases, thus early recognition of tumor formation is an important question during the follow-up and care of patients. The efficacy and significance of currently performed surveillance cystoscopies and random biopsies were questioned, in accordance with current data of the literature from the last 10 years. We re-evaluated the adequacy of our protocol by the re-evaluation of the patients with the longest follow-up duration, a minimum of 10 years. Surveillance cystoscopy alone does not seem to be feasible to detect the development of malignancies in time. Indication, based on specific symptoms related to tumor formation, would result in a higher chance of success to detect tumors in the augmented bladder, so searching for the specific symptoms bears an essential importance.

Several human and animal studies exist to reveal the pathogenesis of tumor formation in the augmented bladder several human and animal studies are exist. The phenomenon of urothelization of the intestinal segment used for augmentation has not yet been fully investigated. Our team wanted to develop an animal model in which this phenomenon can be explicitly examined among the other histological changes.

Summary of novel results and statements:

1. Except Husmann's and our group's findings, we did not find any self-controlled evaluation in the literature which would examine and compare the results from the same patients in two different time periods.
2. According to the results of this study, etiology of bladder dysfunction and the type of augmentation might influence the histological alterations after augmentation cystoplasty.
3. The acid secretion of the gastric wall, the mucus production of the colonic mucosa, and the anatomical construction of the augmented bladder might have an impact on the localization and the type of long-term histological alterations.
4. Metaplastic changes are more common on the mucosa of the native bladder after reconstruction of bladder exstrophy in case of colocystoplasty.
5. Bacterial colonization does not influence the histological appearance of the mucosa of the augmented bladder at the time of biopsy.
6. In the follow-up protocol, symptom related indication of cystoscopies should be preferred instead of biannual surveillance cystoscopies.
7. According to our findings, urothelial replacement of the bowel epithelium could be caused by the metaplastic transformation of columnar cells into urothelial cells.
8. The pattern of metaplastic transformation on the ileum after ICP in rats suggests the inductive effect of the adjacent urothelium and the constant stimulus of the urine to the bowel epithelium.
9. The rat model and the method of histological preparation seem to be effective to investigate the long-term complications as well as the histological changes along the mucosa in rats after bladder augmentation.

Further plans for the clinical and animal studies:

Because of the silently developing complications, the follow-up of patients with augmented bladder due to congenital urinary bladder dysfunction has to be continued. The high complication rate may call for frequent surveillances. The prospective follow-up of the patients is getting more difficult by their aging, as they are disappearing from the pediatric follow-up. Proper transition of the patients to the adult urology care and the creation of a national register are both parts of our most important future plans.

Parallel with the patient care, further studies on the rat model related to the histological changes are needed. Our findings on the ileal mucosa of the augmented urinary bladder in rats should be compared with our previous human immunhistology studies, where cell specific mucin glycoproteins were examined after bladder augmentation. Possibly, the bowel origin of newly developed urothelial cells should be proven by cell specific immunhistological studies.

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