HISTOPATHOLOGICAL EXAMINATION OF THE VESICOURETERAL JUNCTION AND THE LONG-TERM EXAMINATION OF SUBURETERIC INJECTION TREATMENT IN VESICOURETERAL REFLUX

- Human and animal experimental investigations -

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Clinical significance of vesicoureteral reflux (VUR)

In infancy and childhood vesicouretral reflux (VUR) from the urinary bladder into the ureters and the kidneys can often be detected in the background of uretral infections and pyelonephritis (PN). Undetected or untreated VUR may lead to kidney insufficiency in the long-run. Clinical observations confirm that the course of VUR may vary with age. In infanthood and early childhood reflux is often found to cease spontaneously without any treatment, it maturates, while in some cases despite recognising and treating VUR in time further damage of the kidneys cannot be stopped. No explanation for the differences in the factors influencing the course of the disease can be given by histological and molecular genetics research on the topic.

PN often appears as a consequence of VUR since the urine reflux from the urinary bladder to the kidneys delivers bacteria into the upper urinary tract. PN may lead to the damage of the parenchyma of the kidney, which results in deteriorated kidney function. The treatment strategy of VUR may be determined by the discovery of the factors influencing the course of the disease and the long-term successful treatment. In the view of all these effective treatment methods may be applied which may contribute to the prevention of kidney insufficiency due to VUR, dialysis treatment or kidney transplantation.

The role of ureter motility in the development of VUR is not clear. It is not known if ureter motility disorder resulting from VUR is primary or secondary, i.e. if motility disorder leads to the development of VUR or the pathological motility is a consequence of VUR.

Untreated or undetected VUR can seriously damage the kidney affected and reflux nephropathy (RN) may appear. Progression of RN may lead to hypertension or, in the case of bilateral kidney involvement, to prerenal uraemia or uraemia.

Factors responsible for the development of RN are not fully known. Damage of the kidney affected may occur as a result of long-term persistent VUR or RN may not develop at all. In a certain number of cases RN shows a fast progression and, at a young age already, severe kidney failure may develop even due to short-term VUR. The severity of VUR play a crucial role in the development of RN but it is doubtful whether it is VUR only, which determines the appearance and progression of RN. In the past decades researchers focused on such immunological processes, which can genetically determine and individualise the intensity of the inflammatory responses given to pathogens, consequently having an effect on the severity and progression of the damage of the kidney parenchyma.

Treatment of VUR

The primary aim of VUR treatment is the prevention of infections affecting the kidneys especially the development of PN. Recurrent upper urinary tract infections may result in kidney failure and the development of kidney parenchyma scars, i.e. they may lead to RN. In the case of VUR, depending on the severity of the disease, the age and the condition of the kidneys, there are several methods for treatment. When severe, high-grade (gr. IV-V.) reflux can be detected in infanthood temporary relief of the kidneys may be necessary by surgical treatment. In this case vesico-cutaneostoma is prepared. Eliminating VUR the measure of the widening of the ureter and the renal cavity as well as the frequency and the severity of urinary infections may decrease or cease. In severe VUR the duration of the relief of the kidneys is usually 6-12 months. Following the disappearance of the widening the stoma can be closed. In the case of persistent VUR, at the time of closing the stoma, ureter(s) neoimplantation into the bladder is performed. The treatment of VUR is also possible by using a conservative treatment method, which is a long-term and low-dose antibiotic treatment, i.e. a chemoprophylaxis. This conservative treatment method is usually applied in a young age, in a low-grade VUR (I-III) and when the kidneys do not show RN and are in a good condition. The prophylaxis, under strict nephrological control, is generally recommended for a year. After this repeated MCU or IRC examination is performed to detect the severity or the disappearance of VUR. The results determine the possibilities for further treatment. When conservative treatment does not result in the disappearance or maturation of VUR, or despite the adequate conservative treatment there are serious infections, PN develops, which can be managed by surgical treatment. Primarily, the minimal invasive *endoscopic subureteric injection treatment (STING)* should be chosen, which, following an unsuccessful conservative treatment, is used as an alternative of the open surgical intervention. Its main advantages are that it is an out-patient treatment, it does not cause any pain, it is less demanding for the children’s organism and, when it is unsuccessful, it can be repeated. Moreover, when it is required an anti-reflux

operation can be performed. Open surgery can be performed when RN does not respond to the injection treatment or when it reaches a severe grade (IV-V). In this case the ureter, forming a new submucous canal with appropriate length, is implanted into the bladder again (ureter neoimplantation).

STING

STING, as a minimal invasive treatment method, was developed by Puri and O'Donnell in 1984. This semi-conservative treatment method has become well-known world-wide. At the beginning teflon was used as a material for injection. Later on other materials, such as Macroplast or Deflux, were introduced. During the intervention urethro-cystoscopy is performed in general anaesthesia. The cystoscope can show the orifice of the ureter reflux. Through the cystoscope, a needle fixed to a thin metal or plastic tube is led into the bladder. The needle is stuck in the submucosa of the ureteral orifice and, through the thin tube fixed to the needle, 0.2-0.6 ml injection material (teflon, Macroplast, Deflux) is injected under the mucosa. The injected material forms a sphere-like, bulging depot under the submucosa, which raises and narrows the ureteral orifice, increases the length of the intravesical ureter and prevents the urinary reflux from the bladder towards the upper urinary tracts.

Recently some technical alterations in the injection treatment have been recommended, as a consequence of which the efficacy of the treatment improved. The Hydrodistension Implantation Technique (HIT) has been introduced, by the help of which, using a stream of fluid, the ureteral orifice can be opened thus making the injection in the upper, i.e. intramural, part of the ureter possible. A drawback of STING treatment is that it is performed in general anaesthesia. On the other hand the advantages are that it is not demanding for the children's organism, it is a short intervention causing a minimal post-operative pain and it does not require hospitalisation for several days. The results show that as a consequence of one single injection VUR disappears in 75-80% on average, while after the second one it ceases in 94%.

The role of C-kit positive cells in ureter motility

Those cells are called c-kit positive cells, on the surface which c-kit proto-oncogene or tyrosine kinase receptors can be detected. The tyrosine kinase enzyme plays a major role in cell differentiation. The c-kit receptor is also known as CD117 (cluster of differentiation). ICC showing c-kit positivity was discovered and described first by a Spanish neurohistologist, Ramon y Cayal in 1893. ICC can be found in the entire length of the gastrointestinal tract. It is also called as a pacemaker cell because electrophysiological examinations show that it produces and transmits slow electrical waves. From morphological aspects the cells are bipolar with two dendritic processes, little cytoplasm and an ovoid nucleus.

What stands in the physiological background of the peristaltic movement of the ureter is still unknown. In vitro investigations show that following the denervation of the ureter the autonomous pacemaker activity of the ureter can be detected.

Electrophysiological examinations showed that the starting point of the pacemaker activity of the ureter is the proximal area of the renal cavity. Pacemaker potential was measured both in the upper and the middle and the lower section of the ureter. It seems that two kinds of cells play a major role in the pyeloureteral peristalsis. One of them includes ICC or ICC-like cells producing low wave potential changes and the other includes atypical smooth muscle cells with high frequency. The stimuli are transmitted from the atypical smooth muscle cells providing high frequency directly through the ICC cells. to the muscles. As a consequence of the interaction between the two cell types the frequency of the peristaltic wave decreases from the pyeloureteral process towards the distal ureter. Electromicroscopic examinations of the ICC showed that in some cases these cells have several processes and have connections with some smooth muscle fibres and nerves.

The disorder of ureter motility, the lack of ICC or decrease in the number of ICC can be suspected in the background of hydroureter development in certain diseases, such as non-obstructive hydronephrosis. In pyeloureteral stricture a significant drop in the number of ICC was detected. In addition, in vesicoureteral reflux remarkably less ICC can be detected in the VUJ.

Cells showing c-kit receptor positivity but having morphology different from the ICC can be detected throughout the entire length of the uropoietic tract. These cells are histiocytes or mastocytes, which are spherical in shape and contain granules within their cytoplasm.

The granulomas contain some protein-like materials. They can be detected in the lamina propria of the ureter and the VUJ, in the pyelon and also in the walls of the bladder. Their primary role is to give inflammatory or allergic immune reactions. During the activation of the cells active proteins are released from the granules found within the cytoplasm. In the granules of the mastocytes there are several peptides known, such as histamines, prostaglandins, leukotrienes and cytokines. Although the role of mastocytes in ureter motility is not known it can be suspected that the materials released by paracrine mechanism may have an effect on the smooth muscles of the ureter, i.e. on ureter motility. The structural and functional relationships between the mastocytes and the ICC, and also their influence on motility in the gastrointestinal tract have already been demonstrated.

The role of the toll-like receptor 4 (TLR4) in the elimination of the uropathogen

The immune system has to recognise the harmful antigens and has to separate them from its own useful antigen store in a way it becomes and remains tolerant to the latter ones. The immune system can be divided into two main parts. One is the so called inborn part providing natural immunity and the other is the acquired part responsible for the adaptive defence mechanisms.

The TLR, which is primarily responsible for detecting pathogens, is a molecule (13 are known at present) coded by the egg cell as part of natural immunity. It belongs to the family of the so called pattern recognition receptors (PRR) and it recognises the molecular structure or structures expressed on the cell surface, i.e. the Pathogen Associated Molecular Pattern (PAMP). Lipopolysacharids (LPS), bacterial lipoproteins (BLP), peptidoglycans (PGN) and lipoteichol acids (LTA) belong to the primary PAMP-ligands. Through the individual TLR receptors other and other microbial ligands activate the given cells (with some overlaps). The different ligands generate partly common, partly private gene expressions. The TLR signalling pathways activate the common transcription factors. Although different TLR signals involve similar or identical molecules in the process during their activation, they can also activate immunological responses different from one another and specific to the given TLR. Recently research has been focusing on each TLR, especially on TLR4. The significance of TLR4 can also be explained by the fact that it functions as an LPS receptor. The importance of sepsis caused by Gram-negative bacteria make the studies of immune reactions given to endotoxins relevant. The biochemical and molecular biological processes determining and influencing the intensity of TLR4-LPS immune reactions are also important from this respect. They determine the efficacy of natural immunity against Gram-negative pathogens. Due to the mutation having an effect on the structure of PRR, there is no cell activation by the LPS. Consequently, those individuals who suffer from this are prone to develop infections caused by septic bacteria. *The studies on TLR4 polymorphism (genetical variant coding individual*

patterns) are remarkable because they give reasons why individuals respond with a less intensive (e.g. air way) reaction given to inhaled toxins than others. The expressed TLR4 polymorphism is associated with a reduced response activity given to LPS, which, consequently, results in a more moderate irritative (e.g. air way) reaction. Due to the decreased cytokine production, however, the resistance to endotoxins also becomes weaker, which may be the source of frequent and severe infections.

The TLR4 can be detected in the uroepithelium of the pyelon, ureter and urinary bladder. It is also expressed on the surface of monocytes and histiocytes. It is responsible for the recognition of the bacterial endotoxin part of the uropathogen LPS as well as for the activation of the immune response and the inflammatory reaction. The role of TLR4 in VUR is not known. Clinical observations show that RN does not develop in a certain number of patients suffering from VUR despite the long run of the disease, while progressive renal failure, RN, develops in a short time in other patients suffering from VUR. In several cases further renal damages cannot be prevented even by adequate treatment of VUR. It is still unknown what factors are responsible for this diversity in the course of disease. It can be suspected that individual immune reactions, the intensity of the inflammatory reaction to pathogens and the epithelial reactions developing due to the inflammatory response can lead to kidney damages in different extent. The TLR4 and the immune response activated by itself play a major role in the recognition of uropathogens. Further studies are needed to show whether the individually different expression and the polymorphism of TLR4 may be responsible for the diversity of the course of VUR, which may have an influence on the development of RN.

Main goals

Animal investigations

The study of the effect of VUR developed by open surgery in Vietnamese pot belly pigs on the c-kit positive cells playing a role in ureter motility.

Human investigations

1. The study of the efficacy of STING-treatment on the long run. The ultra sound follow-up of teflon depot in order to study whether the teflon depot has an effect on the success of STING-treatment, the occurrence of urinary infections and the progression of RN.

2. The demonstration of the TLR4 expressed on the epithelium located on the VUJ part of the patients with VUR having undergone an antireflux operation. The study of the TLR4 expression and the relationship between the development of RN and its progression.

Patients and Method

1. Development of VUR in Vietnamese pot belly pigs. The demonstration of c-kit positive cells in the VUJ of Vietnamese pot belly pigs

We developed VUR by open surgery in 10 Vietnamese pot belly pigs. The operations were performed in intratracheal narcosis on 3-4-month old, 10-15 kg in weight female Vietnamese pot belly pigs. The animal investigations were carried out with the consent of the Regional Research Ethics Committee of the Medical Health Care Centre at the University of Pécs, Hungary (licence number: 04324/2006).

Following a lower median laparotomy the abdominal wall muscles were separated in the midsaggital line. Then we approached to the urinary bladder, which was opened saggitally. The ureter orifices were situated deeply on the area of the bladder neck. The ureter orifice on the left side was incised by a pair of straight scissors in the length of 8-10 mm in the protection of a metal probe. Turning out the edges of the incised ureter we sewed it out to the bladder mucosa with some sutures using a 5/0 Vicryl Rapide thread. The purpose of the ureter incision was to shorten the intramural portion of the ureter. VUR developed because of maintaining the ureter orifice open by sewing it out to the bladder mucosa. The bladder was closed by chain sutures (4/0 Vicryl thread).

A 14 Ch transurethral silicon catheter fixed to the bladder neck was placed in the bladder.

The bladder catheter was removed on the 3rd post-operative day. Following surgery MCU was performed in order to control the efficacy of the intervention, i.e. to demonstrate the development of VUR. During the examination Iopamiro-300 was used as a contrast material.

The MCU was repeated 4 weeks following the operation. Only those animals were included in the study, in which VUR could be detected even 4 weeks

after the intervention. During the repeated MCU the pigs were sedated with a combination of some premedication agents used for surgery. The bladders were filled transurethrally.

The severity of VUR was grade II-III in every case. In order to provide for antibiotic prophylaxis 25 mg/kg of body weight of ampicillin was mixed into the food of the animals twice a week. During the 6-month follow-up, to exclude the presence of any infections, three bacteriological examinations of the catheterised urine were performed. The pigs were killed 6 months following the surgery. The non-refluxing ureter on the right side and the refluxing ureter on the left side together with the VUJ process were removed. Following a 4% paraformaldehyde fixation the tissues were embedded in paraffin and sections of 8μ sickness were prepared for the purposes of histological and immunohistochemical processing. Immunohistochemical procedures were applied to demonstrate the c-kit receptors. As primary antibodies mouse monoclonal CD117 antibodies (Novocastra, Newcastle upon Tyne, United Kingdom) in a 1:40 ratio solution and rabbit polyclonal CD117 antibodies (Santa Cruz Biotechnology Inc. Santa Cruz, California, USA) in a 1:200 ratio solution were used. As secondary antibodies and also for signalling the receptors Envision+System DAB (Dakocytomation, Glostrup, Denmark) reagent solutions were applied.

Following the immunohistochemical signalling haematoxylin staining was done to be able to separate the cells more easily.

For the purposes of negative samples (controls) the non-refluxing (not incised) right ureter and the VUJ of the same pig served, on which the same immunohistochemical examinations were performed. Using a light microscope the number and distribution of c-kit positive cells were compared in VUR and non-VUR ureters. The samples were dissected from the right and left distal ureters and VUJ sections of each animal, from 5-5 different, non-adjacent parts. Ten adjacent fields of 1 mm^2 were examined. The evaluation of the samples was performed by two histopathologists independent from one another.

2. Detection of TLR4 on the epithelium of the distal ureter and the VUJ in healthy subjects and in those affected by VUR (human investigations)

For the purposes of our study we used the distal ureter ends and VUJ sections removed due to VUR by antireflux surgery (Cohen, Politano-Leadbetter) in Hungarian paediatric surgery centres. The samples were put in liquid Nitrogen right after the sampling and stored at -80 ºC. Altogether 16 children's distal ureters and VUJ sections were examined. Each patient involved in the study suffered from unilateral VUR. On the basis of severity distribution reflux was grade III in seven patients, grade IV in five patients and grade V. in four patients. At the time of the surgery the patients' average age was 4.6 years (6 months – 12 years). Prior to the surgery, the patients received permanent antibiotic prophylaxis. They did not undergo any other treatments (e.g. STING) and they did not have any infection a month before the surgery. Prior to the antireflux intervention DMSA examination was performed in all the 16 patients. The presence of RN was detected in eight patients. The control samples (8 ureter ends) were gained partially from young kidney donors (6 samples) and partially from children died in accidents (2 samples). The study was carried out with the consent of the Regional Research Ethics Committee of the Medical Health Care Centre at the University of Pécs, Hungary (licence number: 04324/2006).

The samples were divided into three groups. The first group involved those samples, in which RN could be detected as a consequence of VUR (8 ureters – RN positive). The second group contained those patients' samples, in whom, besides VUR, DMSA did not show RN (8 ureters – RN negative). The third group was consisted of the control samples (8 ureters). From the distal segments of the ureters and from the VUJ sections in each group 15-15 dissections of 8µ thickness were prepared using the cryostat sectioning technique.

The immunohistochemical examinations were carried out by using TLR4 polyclonal rabbit primary antiserum (Santa Cruz Biotechnology Inc. Santa Cruz,

California, USA). PBS solution was applied for thinning and the thinning ratio was 1:300. The sections were incubated for 12 hours at +4 ºC. The Envision+System DAB (Dakocytomation, Glostrup, Denmark) solution was used for staining. We did haematoxylin staining in order to make the separation of the cells easier. To demonstrate the specific immune reaction the immunohistochemical staining was also done without the intubation with the primary antibody.

The samples were examined by light microscope. We examined whether the TRL4 expression appears on the epithelium of the ureter. The intensity of the TLR4 expression was compared in the digital pictures taken from the samples of the three groups. The immunohistochemical examinations showed TLR4 positive spherical cells even on the subepithelial surface of the ureter.

To find out the origin of the cells double immunofluorescence staining was performed using c-kit monoclonal mouse and TLR4 polyclonal rabbit primary antiserum. The light microscopic analysis on the 5-5 sections dissected from different segments of the distal ureter was carried out and evaluated by two histopathologists independent from one another.

3. Examination of the detectabilty of teflon-depot, the occurrence of urinary infections and the development of RN during the long-term follow-up of STING treatment

At the Department of Paediatrics at the Paediatrics Clinic of the University of Pécs, Hungary STING treatment was performed in 136 children suffering from VUR between 1989-2000. Six weeks following the intervention US examination was carried out and 6 months after the intervention MCU was performed. Only those children were included in the long-term study, who attended nephrological check-ups on a regular basis, in whom the US examination six weeks after the injection treatment showed teflon-depot and six months following the STING treatment MCU demonstrated the disappearance of VUR. The average length of the follow-up was 10.4 years (4-14 years). Out of the 136 patients 99 (143 ureters) met the criteria. At the time of the STING treatment the severity distribution of VUR in the 143 ureters were the following: grade I: 8, grade II: 24, grade III: 78, grade IV: 30 and grade V: 3 (figure 1). In grade I VUR injection treatment was performed only in those patients who suffered from bilateral VUR and the tratment was indicated due to the more severe contralateral VUR. Bilateral VUR developed in 47 cases. The boys-girls distribution was 78:21. During the long-term follow-up, due to the detectabilty of teflon-depot by US, the patients were divided into two groups: **depot (+)** – the teflon-depot can be detected in the bladder **depot (-)** – the teflon depot cannot be detected in the bladder. We examined whether there is a relationship between the development or disappearance of teflon-depot and the recurrence of the reflux. We also examined the frequency of urinary infections during the treatment (significant bacteriuria, PN). During the long-term follow-up DMSA examination was also performed. In those patients who had DMSA prior to the STING treatment there was an opportunity to examine the percent alteration of the distribution of newly developing parenchyma scars and the renal function. RN was diagnosed when DMSA clearly showed the parenchyma scars and the refluxing kidney function was below 25% compared to the total kidney function. *Chi-square* test was used for the statistical analysis.

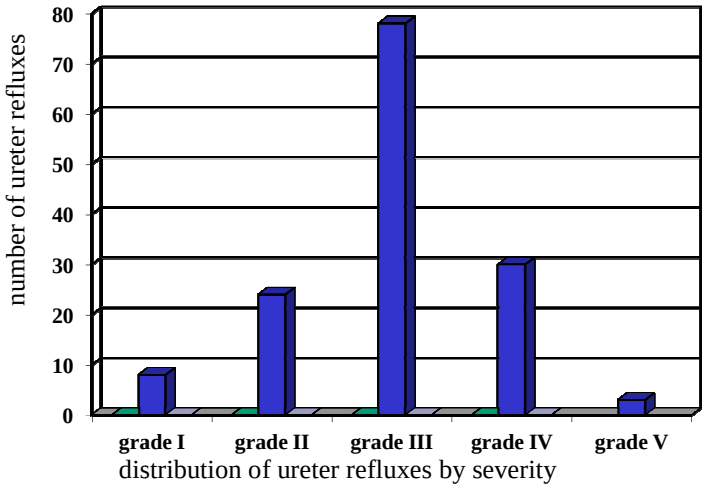

Figure 1
Distribution of ureter refluxes by severity at the time of the STING treatment

Results

1. The effect of VUR developed surgically in Vietnamese pot belly pigs on the c-kit positive cells in the VUJ

Our examinations showed the c-kit positive cells both in the samples with reflux and in the control samples. However, two morphological different c-kit positive cell groups could be differentiated.

The ICC or ICC-like cells with two processes were put in the first group. These cells were found in the subepithelial layer of the VUJ and between the longitudinal muscle fascias.

The second group included the spherical cells, in the cytoplasm of which granules could be seen. These cells were also located in the epithelial layer and between longitudinal muscle fascias and, based on positivity and morphology they corresponded to mastocytes.

Investigations using polyclonal and monoclonal antisera yielded similar results in relation to the detectabilty and arrangement of c-kit positive cells. Refluxing and control ureters showed neither morphological nor size-differences in the two cell types.

The examinations showed that the number of mast cells increased in the VUJ section of the ureters with VUR compared to the control samples with VUR. On the other hand the number of the ICC remarkable decreased in the VUJ section of the ureters with VUR compared to the control samples with VUR (table 1).

Table 1 presents the difference in number of mast cells and ICC in VUR and non-VUR ureters.

	VUR	CONTROL
CAJAL CELL	**+/-**	**++++**
MAST CELL	**+++**	+

Table 1
Changes in the number of Cajal cells and mast cells in the refluxing and non-refluxing (control) ureters of Vietnamese pot belly pigs. The measure of alteration is indicated by +/- signs.

2. The difference in the expression of TLR4 in healthy and VUR VUJ and ureter epithelium (human investigations)

TLR4 expression was detectable in the ureter epithelium, both VUR RN positive and RN negative, in the non-VUR control group. In the RN positive and RN negative groups in the subepithelial layer and between the longitudinal muscle fascias spherical cells containing little cytoplasm showing TLR4 positivity could be detected. No TLR4 positive subepithelial cells could be found in the control group. The intensity of the TLR4 expression was identical in the ureter epithelium of the control and RN negative groups. *In the RN positive group the TLR4 receptor expression of the ureter epithelium was significantly more intense as compared to the RN negative and control groups. The double immonofluorescent staining of the TLR4 positive subepithelial cells detected in the RN positive and RN negative samples demonstrated that identical cells also show c-kit positivity. Cells showing TLR4 positivity and c-kit positivity at the same time are regarded as mastocytes on the basis of their morphology. The number of subepithelial mast cells showing double receptor positivity is higher in the RN positive group than in the RN negative group.*

3. Detecting teflon-depot, the incidence of VUR recurrence, the appearance of urine infections and the progression of RN in the course of long-term follow-up of STING treatment (human investigations)

In the course of the 10.4 year-long follow-up in 65 ureters of 43 patients teflon-depot could be detected with US (depot (+)), while in 78 ureters of 56 patients no teflon depot could be detected (depot (-)). In 17 of the 143 ureters investigated reflux recurred (11.9%). The recurrence of the reflux was detected only in the depot (-) group. In the recurrence o VUR significant difference could be found between depot (+) and depot (-) groups ($p<0.05$) (table 2).

	Recurrent VUR	**No recurrence of VUR**	**Altogether**
Depot (+)	0	65	65
Depot (-)	17	61	78
Altogether	17	126	143

Table 2
The recurrence of VUR in depot (+) and depot (-) groups ($p<0.05$)

In the course of the follow-up the severity of VUR was the following: grade I in 6 patients, grade II in 7 patients and grade III in 4 patients (figure 2). In one case the recurrent VUR was bilateral. Comparing the severity of recurrent VUR with that of VUR detected prior to the treatment, the grade was identical in 6 patients, higher in 2 patients and lower in 7 patients. In the course of the follow-up urinary infection was found in 42 patients, while PN was found in 5 patients. In the depot (+) group significant bacteriuria was detected in 17 patients, while PN was found in 3 patients. In the depot (-) group bacteriuria was found in 33 patients, while PN was found in 2 patients (table 2).

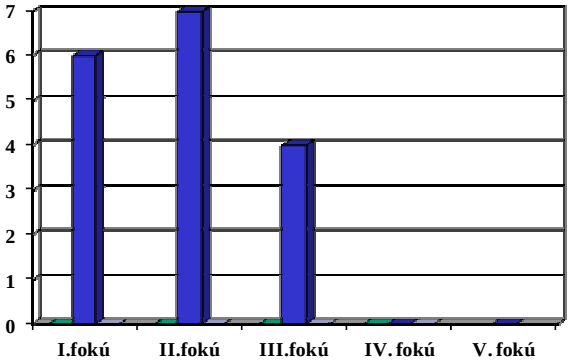

Figure 2
Distribution of the severity of recurrent VUR during a long term follow-up

	Bacteriuria	PN
Depot (+)	17	3
Depot (-)	33	2
Altogether	42	5
Significance	**$p \leq 0.05$**	-

Table 2
Development of bacteriuria during a long term follow-up in the depot (+) and depot (-) groups. The occurrence of bacteriuria in the depot (-) group was significantly higher than in the depot (+) group ($p<0.05$).

Bacteriuria in the depot (-) group was significantly more frequent than in depot (+) group ($p<0.05$), while in the case of PN no significant difference could be found.

DMSA investigation was carried out in 65 patients prior to the injection treatment and following the long-term follow-up. In the depot (+) group 37, while in the depot (-) group m28 patients were examined. At the time of the STING treatment parenchyma-scar could be found in 14 of 37 patients in the depot (+)

group, while scars were found in 7 of 28 patients in the depot (-) group. Following the long-term follow-up in the DMSA examination in the depot (+) group new parenchyma scars were found in 2 of the 37 patients. In the depot (-) group DMSA showed new parenchyma scars in 21 of the 28 patients (table 3).

	DMSA was carried out prior to STING and following long-term follow-up	Parenchyma-scar at the time of STING	New parenchyma- scar appeared	Significance
Depot (+)	37	14	2	-
Depot(-)	28	7	21	**p≤0.05**
Altogether	65	21	23	

Table 3
The appearance of new renal parenchyma-scars demonstrated by DMSA investigations in the long-term follow-up in depot (+) and depot (-) groups. The appearance of new parenchyma-scars is significantly higher ($p<0.05$) in the depot (-) group.

Significant difference between the depot (+) and depot (-) groups was found in relation to the appearance of new parenchyma scars and RN progression ($p<0.05$).

Discussion

1. The effect of the altered expression of c-kit positive cells on ureter motility as a result of VUR developed surgically in Vietnamese pot belly pigs

VUR can be created using our open surgery procedure in Vietnamese pot belly pigs. The experimental model was applicable for the investigation of histological changes due to VUR.

Pyeloureteral and ureterovesicular peristaltics is indispensible for the one-way flow towards the urinary bladder. ICC is a pace-maker cell found in mammals, which has an important role in the peristaltics of the ureter. The cells generate low-wave electric potential, and its alterations can be measured in the urinary tract.

ICC can be detected at each stage of the urinary tract, however the number of cells and distribution can show regional difference between the individual stages. The role of ICC in the coordination of peristaltic waves is not clear. In pyeloureteral junctions in mice ICC was demonstrated to be able to induce contractions in the adjacent smooth muscle cells.

Investigations showed that the nerve fibres necessary for the innervation of smooth muscles do not always reach each individual muscle cell. Presumably the conduction of the stimulus in these cases takes place through the transmission of mast cells through the ion-channels. This function of the mast cells most probably play a part in the motility of the smooth muscle cells located in the walls of the vessels and in the walls of the middle layer of the muscles in the ureter. Nassau *et al* demonstrated that the secretion and the motility of the intestine is influenced by a paracrine mechanism in the mediating substances in the cells and in the mast cells. Research revealed that there is functional relationship between mast cells and ICC.

It was also demonstrated that in the case of surgically created VUR the number of mast cells increased in the VUJ. The pathophysiological role of mast cells in the healthy and pathological ureter motility is not clear.

The present study has demonstrated that in Vietnamese pot belly pigs the number of mast cells increased in the surgically created VUR. Continuous antibiotic prophylaxis was used in our experimental animals so no significant amounts of bacteria were detected in the bacteriological investigations of the urine. Relying on this, we think that the change in the number of mast cells was caused by the surgically created VUR rather than some inflammatory reactions. The mediator substances released from the increased number of mast cells may have an important role in the function of smooth muscle cells located in the wall of the ureter through paracrine or interneural connections. As a result of surgically created VUR the number of ICC decreased in the VUJ, which is likely to deteriorate the motility of the normal ureter. Presumably the increase in the number of mast cells improves the peristaltics of the ureter damaged due to the reduced number of ICC through the mechanisms described above.

ICC or ICC-like cells with dendritic processes and little cytoplasm are also cells showing c-kit positivity. The morphology of the cells as compared to those in the intestinal tract shows minimal difference, the dendritic processes are shorter, which is why they are often called ICC-like cells. These cells occur in high numbers in the septum between the outer and inner sphincters of the ureter. Reference has been made that relying on examinations of humans in certain urological conditions like in the pyeloureteral stenosis or in VUR, the number and distribution of ICC show significant differences. In the VUJ the number of ICC in the case of VUR shows significant reduction as compared to the healthy controls. The differences in the number of distribution of cells, however, are not known to be primary or secondary alterations. *In our animal experimental model in Vietnamese pot belly pigs, as a consequence of surgically created VUR the number of ICC in the VUJ decreased. Since the number of ICC in the non-VUR ureters on*

the opposite side of the same animals was significantly higher, the reduction in the number of cells in the refluxing ureters can be attributed to a secondary alteration due to VUR.

The investigation of the gastro-intestinal tract suggests that the reduction in the number of ICC can be associated with several conditions related to the motility of the intestine. Animal experimental models proved that inflammation affecting the intestine reduces the number of ICC, which, following recovery, gets normalised.

There is experimental evidence that the obstruction caused by the ring placed on the intestine also reduces the number of ICC, which gets normalised after the obstruction is ceased. It is not clear whether the number of ICC gets normalised as a result of similar regeneration process in the uropoietic system or as a consequence of the disappearance of VUR following successful antireflux intervention.

In sum, our animal experiments proved that the expression of c-kit positive cells in the VUJ changes as a result of VUR created surgically in Vietnamese pot belly pigs. The reduction in the number of ICC most probably has an effect on the motility of the ureter and may deteriorate VUR. The role of mast cells in VUR is not clear. ICCs react to the changes in their environment, their number decreases as a result of inflammation or obstruction. Presumably, as a consequence of VUR the increasing number of the mast cells contributes to the modulation of the contraction of the ureter thus improving its motility and compensating for the disturbances in the motility due to the reduction of the number of ICCs. Further investigations are needed to find out which factors and external effects have an influence on the proliferation, differentiation, death or survival of the ICC. The question whether the number of ICC gets normalised following the successful treatment still remains to be answered.

2. The effect of different TLR4 expression in the ureter epithelium on the development of RN in VUR (human investigations)

The role of TLR4 in the protection against uropathogen agents was discussed in the introduction. The investigation of the genetic background of the altered immune response mediated by TLR4 to neuropathogen agents shows that the previously assumed polymorphism and variability are a lot more complex and complicated than it was believed. The occurrence of asymptomatic bacteruria is well known in the clinical practice. The whole population, including children, its incidence can be about 1%. In pregnant women this ratio is 2%, while in the elderly it can be as high as 10%. Investigations showed that in these cases not even the more virulent pathogens cause infection of the urinary tract or severe bacterial sepsis. Therefore, there are still some trends, which suggest that asymptomatic bacteruria does not need to be treated. Animal experiments suggest that the altered expression of TLR4 influences the strength of the response to the infection, which may range between simple bacteriuria and severe life-threatening urosepsis. The present study demonstrated that on the surface of the healthy and VUR ureter epithelium LR4 receptors can be detected. The intensity of the expression of the receptor is stronger in the case of RN than in the group without it or as compared to the control group. In VUR during the flow of the urine in the opposite direction the bacteria get into contact with the epithelium of the ureter and the renal pelvis. TLR4 plays an important part in the recognition and in the induction of immune reaction necessary for the elimination. Based on the different expression of TLR4 it can be assumed that the immune reaction induced through the mediation of the receptor may be of different intensity. The severity of the inflammatory reaction, the consequent fibrosis and the appearance of renal parenchyma-scars and their size depend on the intensity of the immune reaction. It was proved that in the case of a reduced TLR4 expression on the ureter epithelium symptomless bacteriurea

has a higher incidence and in the case of strong expression of the receptor dysurea may occur and PN may appear.

The genetic polymorphism of TLR4 influences the expression of TLR4, which may change the response given by the individual to pathogens. The TLR4 expression varying from individual to individual may explain the immune reaction with different intensity given to the one and the same pathogen. In the case of VUR the altered TLR4 expression demonstrated in the present study, and also as a consequence of immune response of different intensity induced by different expression may have a role in the development of RN. This assumption may be supported by the fact that in the VUJ of RN positive patients and in the ureter TLR4 expression was more intense than in RN negative cases or in the control groups. Both TLR4 and mast cells showing c-kit positivity appearing in the subepithelial layer are most probably cells activated as a result of immune responses induced by TLR4 receptor signals. In the RN positive group the number of these cells is elevated as compared to the RN negative group. No LR4 positive cells could be detected in the subepithelial layers of non-VUR ureters in the control group. This suggests that in the appearance of the TLR4 cells VUR as a pathogenic agent plays an important part.

Our results demonstrate that the severity of VUR is not a an exclusive, unique development of the RN. II-V-grade VUR make it possible for pathogens to get in contact with the epithelium of the ureter or the pyelon. The genetically determined expression of TLR4 and the immune response generated by this plays an important part in the fact that, regardless of the severity of VUR, some patients contract symptomless bacteriurea, while others have recurrent PN or early RN. This may explain the different courses of the disease confirmed by clinical observation. Our results suggest that VUR patients with exclusively bacteriurea do not belong to the high-risk group in relation to the development of RN. The clarification of the immunological factors influencing the course of VUR may provide help for the treating physicians with selecting conservative or surgical

treatment. Our results also suggest that in progressive cases of VUR, which cause early RN, the difference in the immune response mediated by TLR4 receptors is one of the important factors influencing the course of the disease. Further investigations are needed to justify this relationship

3. The effect of the detectabilty or disappearance of teflon-depot on the recurrence of VUR, urine infections and RN progression in the course of long-term follow-up if the STING treatment

The short-term results of STING treatment are well-known. Some authors also deem long-term results. In the present study, no VUR developed on the collateral side following STING treatment. In the course of endoscopic VUR treatment the depot at the ureter orifice is known to contain glycerine and its volume decreases by 50%, even though it can be detected by US. No data were published concerning the long-term detectabilty of the depot and it is not clear either whether or not its absorption or disappearance influences the duration of the successful treatment. In the present study its flowing out through the injection canal can be since 6 weeks following the injecting US examinations always detected teflon-depots. In half of our patients after a long-term follow-up the depot on the injected area could not be detected. The reason for the late disappearance of the teflon-depot is unknown. A possible reason can be the migration of the teflon granules. It is known that the migration depends on the size of the injected particles. Of the injectable substances the size of teflon is the smallest (40μ) so its migration is easier.

In our patients, the long-turn recurrence of VUR is the same as the international data. No significant difference could be detected in spite of the fact that grade I VUR is not considered as recurrence by several authors. Our investigations show that between the lack of depot and the recurrence of VUR there is a tight relationship, the depot has an important role in the long-trm success of the STING treatment since the recurrence of VUR could be observed only in 17 of 75 depot (-) ureters. In addition to the mechanic antireflux effect of teflon most probably some other factors also play a part in the duration of success. It is possible that scars developing as a result of inflammatory tissue reactions on the injection site, the elongation of the ureter and its fixation has a role in the cessation of VUR.

Our long-term investigation show that there is significant difference between the depot (+) and depot (-) groups. No explanation was found to the relationship between the lack of the depot and the recurrent bacteriurea. In the case of VUR the role of recurrent urine infections in the development of RN was proved by several authors. Recurrent urine infection in our long-term investigations occurred only in those, where teflon-depot was missing and recurrent VUR cases developed exclusively in these patients. Recurrent bacteriurea may be a warning signal or accompanying phenomenon of VUR. Patients who typically develop recurrent infections of the urinary tract and in whom the teflon-depot cannot be detected with US require special attention and care. These patients, due to the possibility of the recurrent VUR, belong to a higher risk group.

There was a significant difference between the two groups in terms of the development and progression of RN, i.e. the lack of depot increased the development of new parenchyma scars in the affected kidneys. Clinical observations and also our results demonstrate that successful antireflux treatment slows down, but do not cease in every case, the progression of RN. In successfully treated VUR patients, when US cannot detect depot, we consider temporary (6-12 months) DMSA examinations indicated. In the case of the development of new parenchyma scars and/or progressive RN MCU or RRC is indicated in order to exclude the recurrence of VUR. The lack of depot can be a warning sign for the possibility of the progression of RN even in the case of successfully treated VUR.

Our results showed that in the case of successful STING treatment at the beginning the lack of depot has a long-term effect on the development of recurrent VUR, i.e. it increases the possibility of reoccurrence. Further investigations are needed to understand the reasons of the long-term Teflon-depot absorption. In patients having received successful STING treatment, reappearing bacteriuria develop during the treatment, at the same time, however, US cannot show depot on the area of the injection, so careful follow-up is required due to the possibility for the reappearance of VUR.

Further investigations are needed to study the long-term success of injection treatments performed by using other materials – Macroplast, Deflux - for antireflux treatment and the long-term behaviour of the injected materials.

Pioneer and novel results

1. In Vietnamese pot belly pigs VUR can be created by open surgery incising the intramural section of the ureter. The success of the intervention and its permanency can be increased by fixing the edges of the incised ureter to the mucosa of the urinary bladder by some sutures.

2. In Vietnamese pot belly pigs the increase in the number of c-kit positive mast cells as well as the decrease in the number of c-kit positive ICC are secondary alterations due to VUR. The change in the number and distribution of c-kit positive cells has an effect on ureter motility with high probability. By the reduction in the number of ICCs, the intensity of the pacemaker effect generated by the cells decreases, which may worsen ureter motility and determine the severity of VUR. The increase in the number of c-kit positive mast cells – through the paracrine and interneural relationships – may have an effect on ureter motility due to its direct influence on the smooth muscles of the ureter.

3. The different expression of TLR4 on the ureter epithelium determines the development of RN due to VUR. The expression of TLR4 is more intense in patients developing RN due to VUR.

4. In the group of VUR patients, where RN is detectable the number of TLR4 positive cells increased in the subepithelial layer of the ureter. Double immunohistochemical examinations showed that these cells are mastocytes showing c-kit and TLR4 positivity, which probably were activated through the TLR4 signal. The alteration in the number of mast cells also indicates the individually different intensity of the immune reactions mediated by TLR4, which may determine the intensity of inflammatory reactions, i.e. the development of RN.

5. The teflon-depot detectable in the urinary bladder by US six weeks following successful STING treatment disappears in half of the patients during the long-term follow-up.

6. The lack of teflon depot reduces the long-term success of STING treatment, significantly increases the development of bacteriuria and the recurrence of VUR.

7. During the long-term follow-up of the successful STING treatment the disappearance of teflon-depot significantly increases the progression of RN and the development of new parenchyma scars.

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