ASYMPTOMATIC BACTERIURIA IN TYPE 1 DIABETIC CHILDREN

Doctoral Thesis

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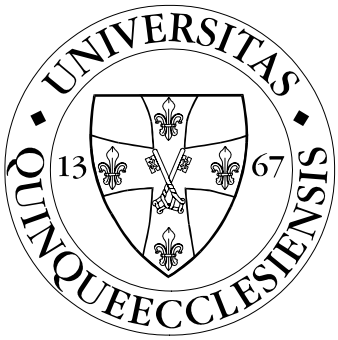

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I. ASYMPTOMATIC BACTERIURIA IN HEALTHY AND DIABETIC POPULATIONS

Urinary tract infections (UTIs) are among the most common bacterial infections in the healthy population. Up to 50% of women report having had at least one UTI in their lifetime. Uncomplicated UTIs occur most often in young healthy adult women and are easy to treat. However, in other patients groups, UTIs can have a complicated course, are more difficult to treat and often recur. Complicated UTIs occur most commonly in patients with abnormal genitourinary tract. However, other conditions such as old age, immunosuppressive treatment, immunocompromised state and diabetes mellitus also predispose to an enhanced susceptibility for the development of UTI with complicated course (1).

UTI is a significant problem in patients with diabetes mellitus because of the multiple effects of this disease on the urinary tract and host immune system. Complicated UTIs associated with diabetes include renal and perirenal abscess, gas-forming infectios, such as emphysematous pyelonephritis and emphysematous cystitis, fungal infections and renal papillary necrosis (2). The chronic consequences of diabetes on the genitourinary system are generalized vascular disease (including renal artery stenosis), diabetic nephropathy, diabetic neuropathy resulting in bladder dysfunction, different degree of glucosuria and abnormal function of the immune system (2). In general, patient history is helpful in the localisation of the infection. The symptoms of lower UTI include dysuria, frequency, urgency, small-volume voids or lower abdominal pain. On the other hand, upper UTI is characterized by fever, nause, vomiting, flank pain or costo-vertebral tenderness (3). Widespread acceptance and application of the quantitative urine culture identified several patient populations who were clinically

asymptomatic but had high prevalence of positive urine cultures. These included pregnant women, individuals with urological abnormalities, patients with indwelling urethral catheters and some diabetic patients. This entity is called asymptomatic bacteriuria (ASB).

I/1. Definition of asymptomatic bacteriuria

Acceptable methods for urine collection include midstream clean-catch, catheterization and suprapubic aspiration. The first method is preferred for routine collection of urine for culture. If there are more than 10^5 colony-forming units (CFU)/ml in a clean-catch urine, there is 80% probability that it is true bacteriuria. If two different samples demonstrate the same specimen at least 10^5 CFU/ml, the probability increases to 95% (4).

The term ASB refers to the presence of two consecutive, freshly voided, midstream, morning urine specimen both yielding positive cultures ($\geq 10^5$ CFU/ml) of the same bacterium, in a patiens without urinary symptoms (5). According to the guideline of Infectious Disease Society of America in asymptomatic men a single isolated specimen in a quantitative count $\geq 10^5$ CFU/ml is sufficient to diagnose ASB (5). If the bacterial count is between 10 and 10^4/ml usually contamination is considered in spontaneous voided urine (4).

It is not well understood why patiens with ASB do not develop symptoms, while the organisms are the same types of bacteria that cause UTI. One possible mechanism is that bacteria with decreased virulence may colonize the urine rather than cause a symptomatic infection (6).

I/2. Asymptomatic bacteriuria in non-diabetic populations

I/2.1. Prevalence

In the healthy population the prevalence of ASB is represented in Figure 1. (5, 7). It is less than 1% in full-term female neonates. Male neonates are more often affected than females (~2%). The presence of bacteriuria in neonates is an indication for investigation to rule out congenital malformations, especially vesico-ureteral reflux. After infancy the prevalence of ASB decreases mainly in boys and in preschool children it occurs in approximately 1% of girls and ASB is very rare in boys (5, 6). In school-age girls the prevalence of ASB increases to 2-5% and it remains in this range during adulthood in sexually active women (5, 8). During pregnancy the occurance of ASB do not change, but the consequences are more serious than in non-pregnant women. In young adult men the prevalence of ASB is below 0.01%. In the elderly, the prevalence of bacteriuria ranges from 17 to 50% in women and 6-34% in men (5, 6).

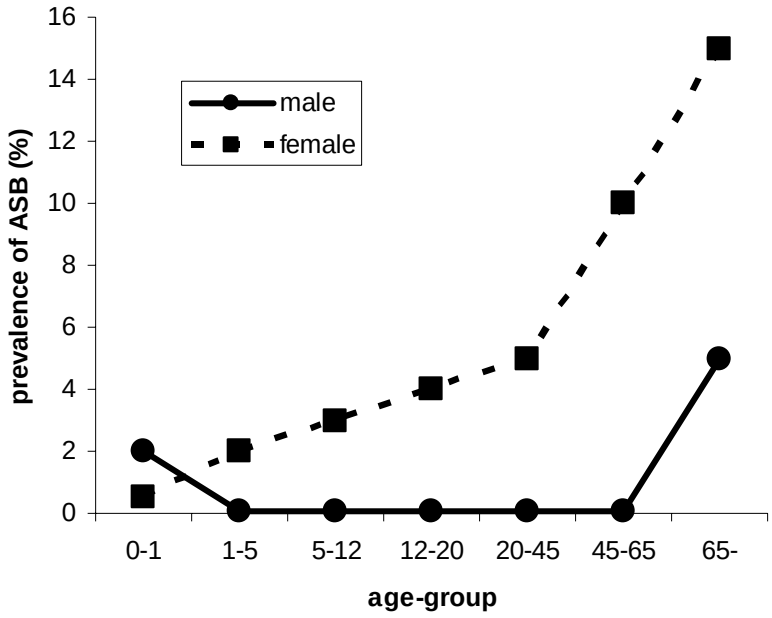

Figure 1.: The prevalence of ASB in healthy women and men (5, 7).

I/2.2. Risk factors of ASB in non-diabetic populations

All neonates are considered to be immunocompromized, therefore they are at a higher risk to any types of bacterial infections. Renal developmental abnormalities which are diagnosed mainly during infancy, but can be discovered in any age-group may predispose to bacteriuria. In non-pregnant young women, ASB is strongly associated with sexual activity. In a prospective study from Hooton et al. the recent use of diaphragm plus spermicide and recent sexual intercourse remained associated with ASB in multivariate analysis (8). In the elderly, some comorbid conditions appear, such as obstructive uropathies, debility, neurogenic bladder, the use of indwelling catheters and incontinence which can contribute to the development of ASB in this population (9).

I/2.3. Immunology and ASB

When an invading organism reaches the uroepithelium it induces production of cytokines, especially interleukin-6 (IL-6) and interleukin-8 (IL-8) (10). IL-6 belongs to the heat shock proteins synthesized by T-cells, B-cells, endothelial cells, fibroblasts, macrophages and epithelial cells and stimulates the liver to produce proteins that are responsible for acute-phase response. IL-8 is a small (~8 kDal) cytokine which is produced by macrophages, endo- and epithelial cells, and it works as an inducer of neutrophil chemotaxis (10). Agace et al. examined the role of IL-8 in the pathogenesis of uroinfection. Women with a history of recurrent symptomatic UTI were colonized with E. coli. Urine samples were collected for IL-8 determination and epithel cells were isolated from the „infected" urine for stimulation by pathogens *in vitro*. There was no IL-8 production before bacterial installation. IL-8 secretion was rapidly (within 3 hours) observed at the onset of UTI, before appearance of the neutrophil influx in the urine.

After bacterial stimulation of the isolated kidney and bladder epithelial cells, it has been shown by immunofluorenscence staining that each type of cells produce IL-8 response locally (11). It also seems that the IL-8 is produced only in the presence of pathogenic agents, because in the case of other inflammatory renal diseases like glomerulonephritis IL-8 was not detected in the urine (12).

IL-8 levels are consistently elevated in the urine of patients with UTI and ASB but they are undetectable in the urine of controls (10, 13). However, at the same time, the IL-8 levels was not elevated in the sera, which also suggests that IL-8 is secreted locally, not filtrated from the blood (13). Systemic lipopolysaccharide (LPS) injections in healthy volunteers did not cause significant increase in the urine chemokine levels, despite a transient increase in the systemic chemokine concentration (14). By immunhistochemistry it was shown that epithelial cells from all parts of the human urinary tract contain IL-8 and this production is constitutive, but the secretion is induced by pathological process (eg.: malignancy, infections) (15). Jantausch et al. have shown that after introducing antibiotics the IL-8 level decreased significantly in children with UTI (16). The mean decrease of IL-8 after antibiotic treatment was 25% after the first dose of antibiotic and more than 80% after at least 2 days of treatment in children with pyelonephritis (17).

IL-6 as an acute-phase reactant also plays an important part in the mechanism of UTI. It seems that IL-6 is elevated in the serum in febrile UTI and its concentration is correlated with C-reactive protein levels. However, in ASB the elevation of IL-6 in the blood and often in the urine, as well was found to be not significant (18, 19).

I/2.4. Genetic studies and ASB/UTI

There are some evidence that the predisposition for UTI is the genetically determined (20). Animal models of UTI have contributed to the understanding of host-bacterial interaction during infectious process. LPS non-responder mice (C3H/HeJ) have general deficiency in cellular responses including neutrophil recruitment that might explain their inability to clear infections (21).

C-X-C chemokines are members of four chemokine subfamilies (Figure 2.) In their molecular structures the first two, highly conserved cysteine residues are separated by a single amino acid nearest to the N-terminal of the protein (22). IL-8 binds to leukocytes by CXC-receptor-1 and -2 expressed almost exclusively on the surface of neutrophils (22, 23). CXCR-2 binds a broad spectrum of CXC-chemokines with low affinity, whereas CXCR-1 selectively binds to IL-8 with high affinity (22, 23). In experimental UTI the neutrophils of IL-8 receptor knockout mice failed to cross the epithelial barrier of the urinary tract and accumulated in the subepithelial tissue (24). In humans, the roles of CXCR-1 and CXCR-2 were studied in many diseases including sepsis, pulmonary-, gastrointestinal- and urinary tract diseases (25-29). Decreased expression or down-regulation of these receptors can lead to impaired neutrophil function, therefore it may play an important role in susceptibility to diseases mentioned above. In a Swedish study, CXCR-1 was examined in children with chronic pyelonephritis. At the time of the measurement of CXCR-1 expression the children were in an infection free period, therefore the CXCR-1 expression was not influenced by acute exacerbation of the UTI. As compared to healthy controls, they had reduced CXCR-1 expression. This finding also suggested a defect of innate host defense mechanism of this subpopulation (29).

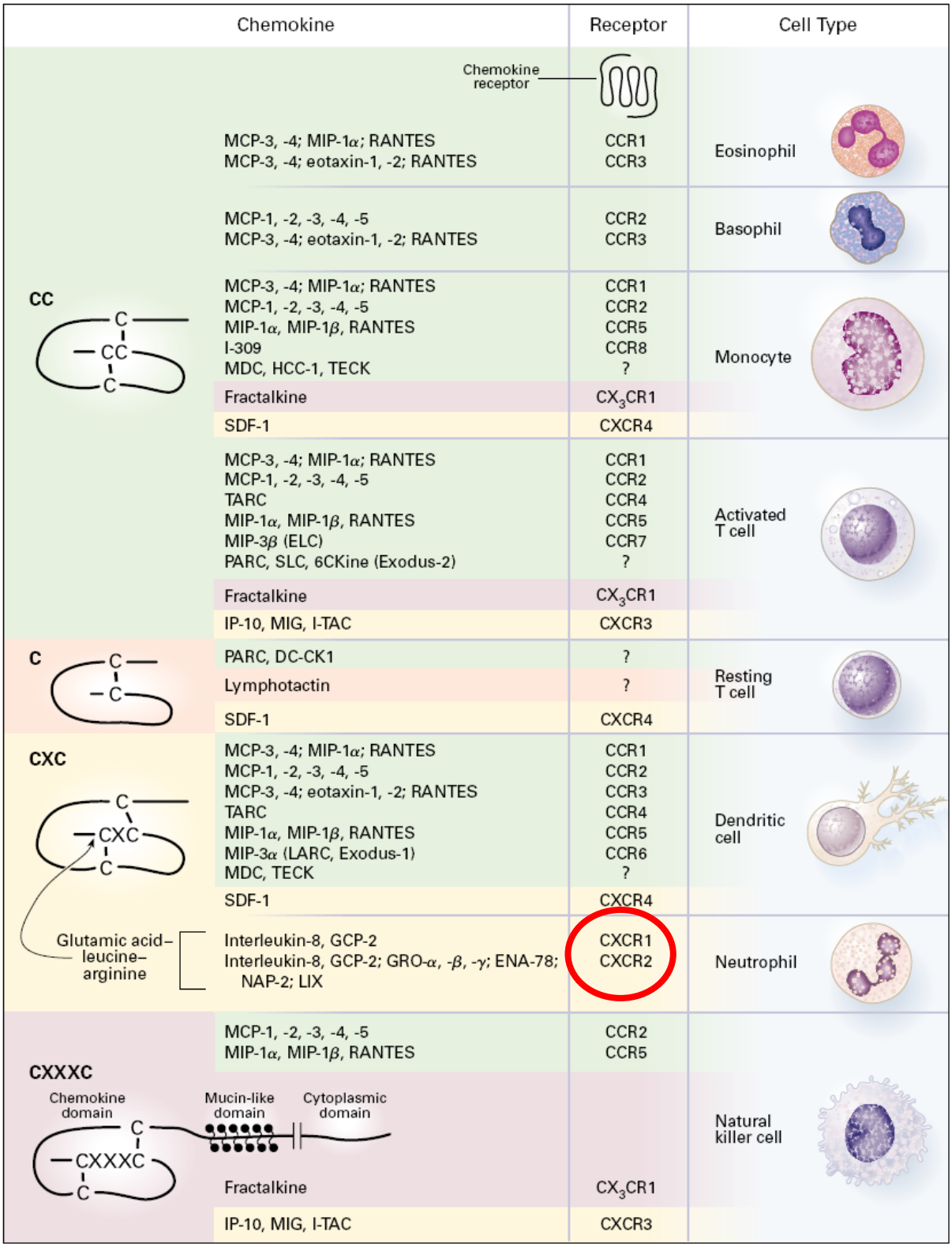

Figure 2.: The members of chemokine subfamilies (22).

In an other study, there was no difference in the expression of CXCR-1 between premenopausal healthy women and those with recurrent UTI (30). Recently, Lundstedt el al. examined the pedigrees of UTI-prone families and the possible association with CXCR-1 expression. They found that acute pyelonephritis was present in 15% in the medical history of family members of children with acute pyelonephritis, but only in 3% of relatives of control subjects. This association was not present in patient with cystitis. They pointed out that CXCR-1 expression was significantly lower in patients and their family members compared with controls and suggested that low CXCR-1 expression could be one of the inherited factors predisposing to acute pyelonephritis (31). There are five known CXCR-1 single nucleotide polymorphisms which are associated with reduced binding of transcription factors and thus resulting in increased susceptibility to UTI (32).

Of course, other candidate genes were also examined which could be involved in the pathomechanisms of UTI. Toll-like receptors (TLRs), one of the main members of innate immunity are expressed on the surface of mucosa and antigen presenting cells. These receptors have pivotal role in recognising highly conserved microbial patterns (such as LPS) and they are working as a pro-infammatory activators. Ragnarsdóttir et al. suggested that children with ASB have decreased TLR4 expression leading to weak mucosal response to urinary pathogens, which could promote asymptomatic state instead of symptomatic UTI (33).

The question, why an infection in the urinary tract progresses to acute pyelonephritis in one individual and causes only asymptomatic bacteriuria in an other one, is very intriguing. The two different pathomechanisms are demonstrated in Figure 3. (34). Invasive microbial strains (including uropathogenic E. coli with P fimbia) in individuals with normal innate immune mechanisms or attenuated stains in hosts with

deficient defense mechanisms causes acute pyelonephritis. If the immune response is normal, renal scarring will not develop (Figure 3a.). Bacterial presence in the urinary tract remains asymptomatic when bacteria with low virulence factors infect the immunocompetent host or if the more virulent pathogens invade the mucosa of a host with decreased TLR4 expression (Figure 3b.) (34).

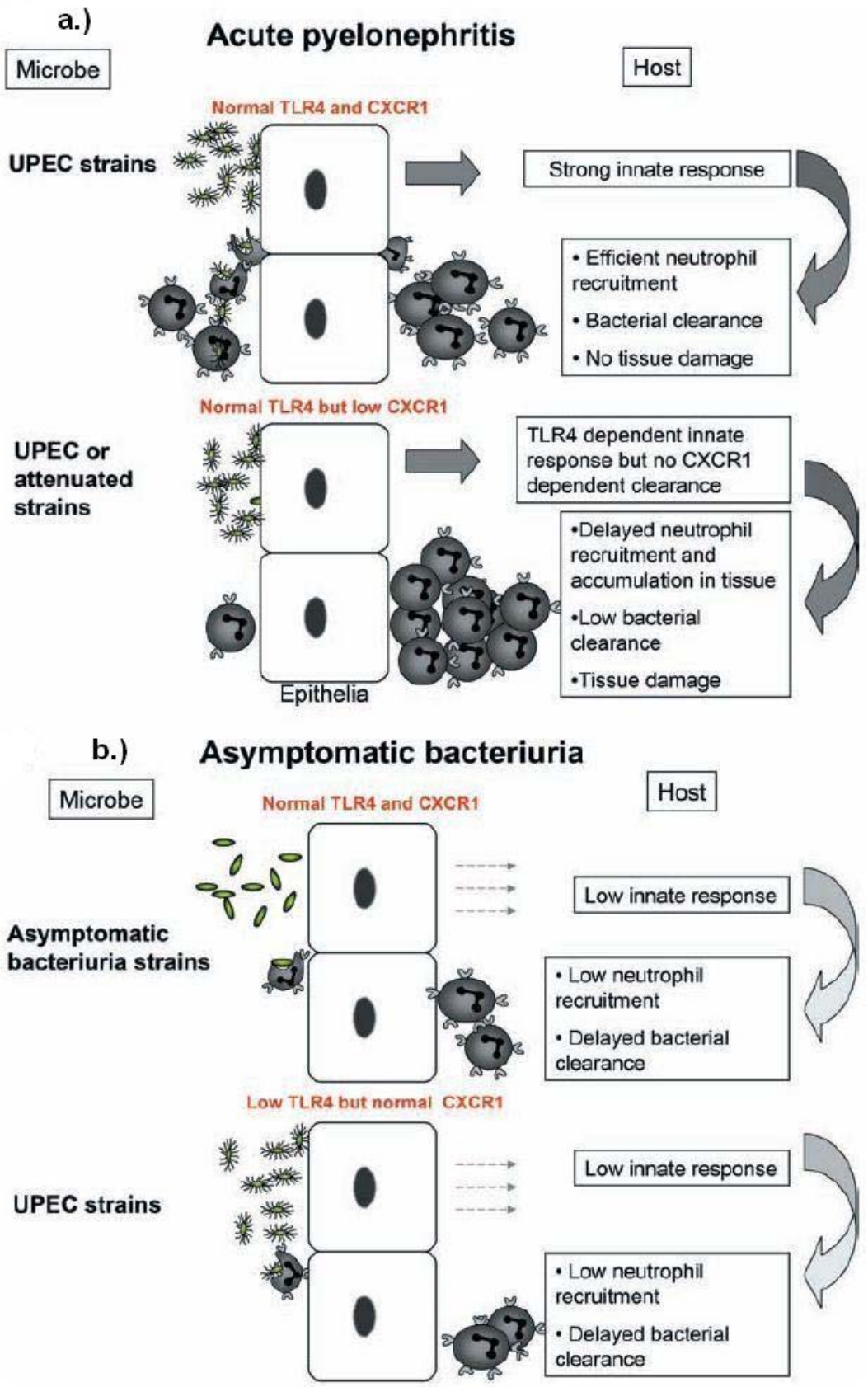

Figure 3.: Different pathomechanism in acute pyelonephritis and ASB (explanation in the text, UPEC: uropathogenic Escherichia coli) (34).

I/2.5. Treatment of ASB in non-diabetic populations

In subjects with normal urinary tract the possibility of renal scarring, declining renal function and hypertension due to ASB is very low (5, 35).

Fifty infants with bacteriuria were followed for 6 years in a Swedish study. Two of the children developed pyelonephritis during the follow-up period and 36 children had spontaneous resolution form ASB. None of the participants developed renal damage or had deteriorating renal concentrating capacity (36).

In a prospective study, ASB was found to be a risk factor for symptomatic UTI in healthy young women. 8% of those with ASB developed symptomatic urinary infection within a week. In contrast, only 1% of non-bacteriuric women had UTI. This difference remained at the same level after 1 month (8). After 15 years of follow-up, 55% of women with ASB at initial screening, developed urinary symptoms at least once. This happened only in 10% of non-bacteriuric women (37). A prospective, randomized, controlled trial examined the effect of a 1-week treatment with nitrofurantoin on ASB and found lower prevalence of bacteriura after 6 month, but not after 1 year after therapy as compared to the placebo group (38). According to the guideline of the Infectious Disease Society of America (IDSA) the treatment of ASB neither decreases the frequency of UTI nor prevents further episodes of ASB in premenopausal, non-pregnant women. Therefore, - they concluded - the screening for ASB is not indicated (5).

In pregnant women, the consequences of ASB are more serious. In most patients, bacteriuria in pregnancy is due to pre-existing periurethral colonization rather than de novo infection during pregnancy (35). Pregnant women have a 20-30 fold risk for developing pyelonephritis during the last trimester and are more likely to experience premature labour or to deliver a low-birth weight infant (5, 6, 37). Antimicrobial

treatment of ASB in pregnany decreased the prevalence of pyelonephritis from 20-35% to 1-4%, therefore the routine screening in early pregnancy and the treatment in positive cultures are recommended (5).

The analysis of the IDSA revealed no benefit of screening and treatment of elderly with ASB living in community (5).

Patients with ASB who undergo urologic procedures associated with mucosal bleeding have a higher risk for bacteremia, therefore, the screening and the treatment is mandatory in these cases (5).

I/3. Asymptomatic bacteriuria in diabetic patients

I/3.1. Prevalence

Urinary tract infection (often asymptomatic) is a frequent clinical finding in adult diabetic patients particularly in women (39). The prevalence of ASB ranges between 9.1-29.3% in diabetic women and between 0.7-11.1% in diabetic men (39). The majority of investigators have reported approximately a three-fold higher prevalence of ASB among diabetic women than among non-diabetic women, but it seems that the prevalence of ASB in diabetic and non-diabetic men is more or less equal (39-41). The majority of data has been collected from patients with type 2 diabetes. Only few studies are available in diabetic children. Two articles are from the 1960's, where unusually low prevalences of ASB (1.6-2%) were reported (42, 43). The last publication in English language evaluating the prevalence of ASB in diabetic children is from 1985. In this study, three of 304 girls and none of the 337 diabetic boys had bacteriuria (44).

I/3.2. Risk factors of ASB in diabetes

The prevalence of ASB among patients with diabetes has been correlated with several clinical characteristics. It seems evident that hyperglycaemia and subsequent glucosuria present optimal circumstances to bacterial colonisation in the urine and uroepithel, which can make diabetic patients susceptible to ASB or UTI. However, it is widely accepted that the development of ASB is not influenced by the type or duration of diabetes (39) and probably the quality of diabetes control measured by glycated hemoglobin (HbA_{1C}) level is not a determinant factor in the prevalence of ASB (39, 40, 45, 46). Few studies reported that elevated HbA_{1C} level was present in the bacteriuric group (47, 48). There are no data available demonstrating that fasting glucose or a randomly measured glucose level (preferably at time of urine collection) was higher in patients with ASB.

There are only *in vitro* experiments which found a positive correlation between the glucose concentration of the urine and the bacterial growth (49). Till now, this study was not confirmed by in vivo data (40, 41).

Microvascular complications are often found after 5-10 years of diabetes duration. Diabetic retinopathy does not seem to increase the prevalence of ASB (39, 50). The results are more contradictory in the cases of micro-, or macroalbuminuria, especially in type 2 diabetes. In type 1 diabetic women, the presence of microalbuminuria is independent from the ASB (40, 48, 50). In a Spanish study, the albumin excretion rate (AER) was examined in women with type 2 diabetes with or without bacteriuria. There was no difference in AER either between the two groups, or before and after treatment of ASB (51). Other studies found the prevalence of microalbuminuria higher among patients with ASB in type 2 diabetes (46). The correlation is clear in the case of macroalbuminuria, which is a risk factor of ASB,

independently from the type of diabetes (40, 46). Neuropathy the third main microvascular complication was found to be a risk factor to ASB in type 1 diabetes (40).

Over 50% of men and women with diabetes have bladder dysfunction, mainly adult patients are affected with this complication (41). However, Hungarian authors have showed with urodynamic studies that asymptomatic diabetic children had longer time to first voiding sensation than that of control children. The average urinary flow was also significantly higher in diabetics than in healthy controls (52). The longer time of the voiding sensation can lead to higher volume of urine fraction which results in increased bladder capacity. Hyperglycaemia diminishes the detrusor contractility of the bladder, therefore the amount of residual urine increases, this retention may promote bacterial colonisation of the urinary tract (53).

Tamm-Horsfall protein (THP) is a glycoprotein in normal human urine produced by cells of the thick ascending limb of Henle loop and the early portion of the distal convulted tubule. It may protect the urinary tract from uropathogens by inhibiting bacterial adherence especially type-1-fimbriae of Escherichia coli (54). It was shown that newly diagnosed type 1 diabetic children had decreased THP concentration which persisted for 1 year after diagnosis (55). The defect of this protective mechanism could also lead to ASB in diabetes.

Several workgroups investigated the the potentially diminished functions of polymorphonuclear leukocytes (PMNs) in diabetes. However, the chemotaxis, the phagocytosis and the killing mechanisms were found to be normal in diabetic patients with ASB (56). In another study, isolated impairment of PMNs function was not detected, but global dysfunction was showed. Before stimulation of PMNs, the adhesion molecule expression, the phagocytosis and killing were more effective and it was

thought that this condition resulted in increased free radical production in the baseline state. After stimulation, however, no further increase was detected in these parameters (57). They found that hyperglycaemia alone was not a determinant factor of PMNs abnormalities.

I/3.3. Consequencies of ASB in diabetic patients

The main question is whether ASB increases the risk of symptomatic UTI in diabetic patients and therefore contributes to the irreversible deterioration in kidney function? Unfortunately, there are only a few prospective, follow-up studies available in diabetic adults and none in diabetic children.

For the diagnosis of ASB two urine cultures are needed. However, after 7-10 days, the second culture will be positive only in about 65% (45). This observation supports the possibility of spontaneous resolution of ASB.

Geerlings et al. found that type 2 diabetic women with ASB had relative risk (RR) of 1.9 to develop symptomatic UTI in a 18-month follow-up period. Similar association was not observed among type 1 diabetic women with bacteriuria (50). In a Spanish study examining type 2 diabetic women and men, ASB was present at baseline in 25.5% and 10.1%, respectively. Over a 12-month period among those with ASB 67.6% of women and 76.5% of men developed symptomatic UTI. The RR for UTI was 4.5 in women and 7.0 in men (58). In the longest follow-up study to date (36 months) 50 diabetic (9 with type 1 diabetes) women with untreated bacteriuria participated (59). After the first year of follow-up 26% of the participants experienced spontaneous resolution or symptomatic UTI and in 36% of them the initially cultured organism persisted. The authors observed that in the group with spontaneous resolution the infecting pathogens were mainly gram positive organisms (78%) and leukocyturia was

less frequently present. The prevalence of ASB decreased rapidly, but after 9 months there was no further change, half of the participants remained bacteriuric. Half of the patients with spontaneous resolution and half of those with symptomatic, treated infection became reinfected again (59).

Neither type 1, nor type 2 diabetic patients with ASB had a higher risk for development of hypertension and there was no deterioration in kidney function (39, 60).

I/3.4. Treatment of ASB in diabetes

Parallel with the follow-up studies, the treatment of ASB in diabetic patients seems to be less and less justified. Studies from the 1990s implicated that a 2-week course of antibiotic therapy was equivalent with a 6-week long regime administered earlier for eradication of ASB. After completing an antibiotic therapy, recurrence was often observed within 8 weeks and it was considered to be mainly reinfection rather than relapse (39). A prospective, randomized trial from Harding et al. examined 105 diabetic women with ASB. Half of the participants were treated with trimethoprim/sulfamethoxazol, the other half received placebo (61). The short-term outcomes of this study were favourable, because after 3 days of treatment 94% of patients was considered as cured in the antibiotic group and only 8% in the placebo group. However, after a 36-month follow-up, the frequency of symptomatic UTI and the time of the first UTI were similar in the two groups. The majority of women (~60%) had no symptomatic episodes, pyelonephritis developed in 11% of placebo group and 6% of antibiotic group (61). It seems that antibiotic therapy cleared the bacteria during the administration period but did not decrease the numbers of symptomatic episodes.

The current approaches to management are different in United States and in Europe. In the US, the treatment is recommended because of the frequency and the

severity of provoked symptomatic UTI. European physicians believe that the benefit of treatment is doubtful (40).

Our own view is that using antibiotics in ASB enhances the possibility of selection of new resistant, more virulent strains. The education of patients to recognise the symptoms of UTI is much more important.

II. AIMS

▪ First of all, we wanted to determine the prevalence of ASB in a large cohort of type 1 diabetic children and young adults and in healthy controls.

▪ The second goal of our work study was to examine the possible differences in some antropometric and metabolic parameters between diabetic patients with and without ASB.

▪ The next aim was to identify the types of microorganisms causing ASB in type 1 diabetic patients and to evaluate the usefulness of the routine screening method of ASB.

▪ Finally, we also wanted to find some immunological factors which could play an important role in the susceptibility to urinary infections in type 1 diabetic children.

1. III. PATIENTS AND METHODS

The aims of the thesis were examined in four large cohorts from the patients of the „Mestyán Gyula" Diabetes Ward, Department of Pediatrics, University of Pécs. The overlap between the cohorts is remarkable in point of participants. We needed some cohorts because the sampling and experiments were done in different time (2002-2006).

It is usual practice in our department to annually hospitalize diabetic children and adolescents for assessment, reeducation and screening for micro- and macrovascular complications. This opportunity was used to collect urinary and blood samples not only for diagnostic purposes but also for research. Newly diagnosed diabetic children, patients with diabetic ketoacidosis and diabetic patients with known renal developmental abnormality were automatically excluded from the studies, except in the case when the effect of ketoacidosis was tested. None of the participants in the study groups had symptomatic infection or fever or received antibiotic treatment during a month before hosptalization. Informed consent was obtained from all participants and the studies were approved by the local ethics committee.

III/1. Characteristics of patients and controls

III/1.1. Participants in „Prevalence of ASB" study

During the observation period (between January of 2002 and October of 2002) 219 diabetic children and young adults were admitted in our department. Ten patients with newly diagnosed diabetes, 4 with ketoacidosis, 4 women who delivered earlier, one being on antibiotic treatment and one who was operated on for a femoral abscess were excluded (n=20) from this study. Three patients with known renal developmental

abnormalities were also excluded. We have to add here that abdominal ultrasonography was not performed routinely in all of our patients.

Altogether 196 eligible subjects participated in this study. 14 of these patients contributed only with one urine sample (nine of these were sterile, three was contaminated, in one 10^5 CFU/ml Streptococcus agalactiae, in another one $>10^5$ CFU/ml Enterococcus species were grown). Two consecutive samples were collected from 182 type 1 diabetic patients, 4 patients were found to have contaminated samples (2 out of 4 had leukocyturia). The 14 diabetic patients with one urine sample and the 4 with contaminated consecutive samples were excluded from further analysis (n=18; 7 males). Their average age was 12.8 ± 5.3 years, the median (IQR) duration of diabetes was 4.4 (2.3-7.7) years.

To assess the prevalence of ASB in type 1 diabetes data of 178 (86 males) type 1 diabetic children and young adults were analysed. Their average age at the time of urine culture was 15.1 ± 5.9 years (range: 4.0-29.4) with 6.2 (3.0-10.1) [median (IQR)] years of diabetes duration (range: 0.5-25.9).

329 healthy schoolchildren / young adults (medical students) were asked to serve as the control group. From 133 out of them only one urine sample was available. One hundred and ninety-six control children provided two consecutive samples, among them two were contaminated. Control patients with single urine samples (n=133) and those with contaminated samples (n=2) were excluded, their average age was 13.6 ± 5.1 years. The average age of the 194 „true" control patients (103 males) was 14.4 ± 5.1 years. The difference in age between patients and controls was not significant (p=0.19).

III/1.2. Participants in „Urinary cytokine" study

137 diabetic children and young adults participated in the study and 178 healthy schoolchildren / young adults (medical students) were asked to serve as the control group. After excluding 4 diabetic patients with contaminated urine the final study group consisted of 133 diabetic and 178 control children. The diabetic group consisted of 66 males and 67 females whose average age at the time of urine sampling was 15.6 ± 5.7 years (range: 3.3-26.6) with 7.3 ± 4.7 years of diabetes duration (range: 0.5-23.4). The average age of the 178 control patients (94 males) was 14.1 ± 4.7 years (range: 3.7-26.6). The diabetic subjects were significantly older than the controls (p=0.013).

III/1.3. Participants in „CXCR-1 expression" study

One-hundred and ten type 1 diabetic children participated in the CXCR-1 study and 54 healthy schoolchildren / medical students were asked to serve as controls. The diabetic group consisted of 43 males and 67 females whose average age (± SD) was 16.0 ± 5.3 years (range: 5.9-29.1) with 7.6 ± 5.5 years diabetes duration (range: 0.5-23.5). The average age in the control group (22 males) was 15.1 ± 6.2 years (range: 6.1-30.1). The differences in the sex ratio (p=0.84), and in the average age (p=0.33) between the groups were not significant.

III/1.4. Participants in „Serum IL-8" study

This study group consisted of 79 type 1 diabetic children (28 males) (age: 15.3 ± 4.7 years with 7.8 ± 5.0 years diabetes duration) and 40 healthy control (19 males) (age: 12.7 ± 5.2; p=0.008 vs. diabetic children).

III/2. Urine sampling and cultures

After cleaning the genitalia and perineum of patients with three gauze sponges saturated by octemidine-dihydrochloride, midstream clean voiding morning urine samples were collected on two consecutive days. The urine samplings were performed (in patients younger than 12 years of age) or supervised (in patients older than 12) by qualified nurses in the same manner and place in both diabetic and control groups. After voiding of the urine the samples were divided into two parts. The smaller fraction of the urine samples was immediately centrifuged at 3500 rpm for 5 minutes and the supernatants were frozen and kept at –70°C until the measurement of urinary IL-6 and IL-8 levels. The remaining urine samples were stored at 4°C (9) until taken to the lab (within 2 hours), where the inoculation was carried out by a calibrated loop on eosin-methylene blue and Colombia blood agar plates (Oxoid, UK). The results were read after 24 h incubation on 37°C. Diabetic patients were hospitalized for 3 days. The measurements of urinary interleukins were performed from the samples obtained on the first day. Urine samples on the second day were used only to diagnose asymptomatic bacteriuria and leukocyturia, but repeated measurements of interleukins were not performed.

III/3. Definitions

To diagnose ASB we used the criteria of the Infectios Disease Society of America (IDSA) (10). Briefly, asymptomatic bacteriuria was defined as the presence of $\geq 10^5$ CFU/ml (colony forming units) of one and the same bacterial species on two consecutive days without symptoms of urinary tract infection (UTI). Contamination was defined as the presence of at least two microorganisms in a sample or different bacteria

on the two consecutive samples. Subjects cultured CFU<10^5/ml with one and the same species were considered to be no-bacteriuria („sterile").

Leukocyturia was diagnosed with two methods. The semiquantitative method by microscope defined leukocyturia as >5 cells/high power field (hpf) at 400X magnification. The dip-slide method (Boehringer, Combur^{10}-Test M, Roche Diagnostics GmbH, Mannheim, Germany) was also used. Leukocyturia was diagnosed if either the microscopic finding or the dip-slide test was positive.

III/4. Measurement of urinary cytokine levels

The measurements were done in Department of Immunology and Biotechnology, University of Pécs. For immunoserological detection of human IL-6 and IL-8 level in urine samples we used optimized commercial OptEIA Sets (Pharmingen USA). Briefly, microtiter wells (NUNC Maxisorp P/N, Cat No. 442404) were coated with 100 µl per well of capture antibody overnight at 4^{o}C. After 3 washing steps, the nonspecific binding sites were blocked with 200 µl/well 10% FBS/PBS buffer for 1 hour at room temperature (RT). After 3 washing steps 100 µl of the standard, control and sample dilutions were pipetted into the appropriate wells and incubated for 2 hours at RT. After 5 washing steps 100 µl of the biotinylated second cytokine specific monoclonal antibody and the avidin-horseradish peroxidase complex (Working Detector) was added to each well and incubated for 1 hour at RT. After 7 washing steps the reaction was developed using ortho-phenylene-diamine (OPD) substrate solution. The absorbance was read at 490 nm within 30 minutes of stopping reaction. To calculate results the mean absorbance of zero standard was subtracted from the mean absorbance of duplicate standards. The standard curve was plotted using the best curve fit of our Labsystem EMS Reader Program MF V2.9-0. To determine cytokine

concentrations of the samples the calculated concentrations were multiplied by the dilution factor.

The concentrations of urinary interleukins were corrected for creatinine, which were measured by the Jaffé method. The final concentration values were given in picogram per milliliter per milligram creatinine. The detection limits were 4.7 pg/mg creatinine for IL-6 and 3.1 pg/mg creatinine for IL-8. The intra- and interassay variability were less than 5%.

III/5. Measurement of CXCR-1

III/5.1. Neutrophil isolation

The measurements were done in Department of Immunology and Biotechnology, University of Pécs. Neutrophils were isolated from fresh anticoagulated (EDTA) blood sample by density centrifugation using Polymorphoprep (Axis-Shield, Oslo, Norway) containing sodium diatrizoate, 13.8% (w/v), and dextran 500 8.0% (w/v) (density, 1.113 g/ml). Five ml of whole blood were layered on 5 ml of Polymorphoprep in a 12-ml tube and centrifuged at 500 x *g* for 30 min at room temperature. The neutrophils in the lower band were harvested using a Pasteur pipette and washed twice with PBS (containing 0.5% BSA).

III/5.2. Staining the cells with CXCR-1 antibody

After the final washing step the viability of the cells was determined using the trypan blue dye exclusion test. Then the cells were resuspended in the same buffer to a final concentration of $4x10^6$ cells/ml and 25 µl of cells ($1x10^5$) were transferred to 5 ml

tubes for staining with fluorescein-conjugated anti-human IL-8RA (CXCR-1) mouse IgG2a monoclonal antibody (R&D Systems Cat.No. FAB330F). Fluorescein-conjugated mouse IgG2a was used as isotype control antibody. The cells were incubated for 30-45 minutes at 4°C, then washed twice in PBS/BSA buffer to remove unreacted anti-IL-8-RA antibody. The cells were fixed in PBS/0.1% PFA buffer and stored in dark before flow cytometric analysis.

III/5.3. Flow cytometry

The samples were run on a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software. 10,000 events were collected from each sample and electronic gating was used to eliminate cellular debris from neutrophil cell population. Fluorescence histograms (in FL-1 channel) were created from this gate and the mean fluorescence intensity of each CXCR-1 labeled sample (referred as mean specific staining Mfs) was compared to the mean FL-1 of the isotype control sample as background (referred to as mean background fluorescence Mfb). Mean CXCR-1 fluorescence was determined by calculating the difference between Mfs and Mfb.

III/6. Measurement of serum interleukin-8

Serum IL-8 levels were measured with an automated immuno-chemiluminescent analyzer (Immulite, DPC) using two-point master calibration and checking the accuracy and precision of the method with bi-level independent controls. The detection limit was 5 pg/ml for IL-8. The measurements were done in Department of Laboratory Medicine, University of Pécs.

III/7. Other laboratory assessments

The daily mean blood glucose level was calculated from an average of 21 measurements (median) (range: 8-43) performed during the hospitalization period of three days by a portable meter using capillary blood (D Cont Personal, 77 Elektronika, Budapest, Hungary), with a measuring capacity between 1.1-25.5 mmol/l.

Glycated haemoglobin was measured by HPLC (Bio-Rad Laboratories Diagnostic Group, Hercules, California, USA; with 4.0-6.3% normal range).

Measurements of urinary and serum glucose (at the time of urine sample collection) were carried out by GOD/PAP enzymatic colorimetric method (Reanal Rt., Budapest, Hungary).

Albumin excretion was measured in 24 h urine sample by liquid-phase immunoprecipitation assay with nephelometric end-point detection (Tubox Microalbuminuria Assay, Orion Diagnostica, Finland) and microalbuminuria was defined as albumin excretion between 30 and 300 mg/day at least in two of three collection performed during the hospitalization period.

The mean value of blood pressure was calculated from an average of 7 measurements (median) (range: 2-22) during admission. Blood pressure was measured by an automated blood pressure meter (Omron M4, Japan).

Information was documented regarding the age and duration of diabetes and BMI was calculated. Pubertal development was assessed according to Tanner stages: prepubertal=Tanner I., pubertal=Tanner II.-IV. and postpubertal=Tanner V.

III/8. Statistics

SPSS for Windows 10.0 statistical software was used (Chicago, USA). Distributions of data were tested for normality by the Kolmogorov-Smirnov test. Mean

± SD are given if the distribution was normal. The distributions of urinary and serum interleukin values were not normal, therefore the median and interquartile range (IQR) were given. The expression of CXCR-1 was given as mean fluorescence intensity and 95% confidence interval (CI). For assessing statistically significant differences Student′s t test (for normal distribution), Mann-Whitney test or Kruskal-Wallis test (for non-normally distributed variables), χ^2 test and Fisher's exact test (for dichotomous variables) and Pearson or Spearman correlation were used. To find the optimal sensitivity and specificity of urinary IL-8 to predict ASB ROC (receiver operating characteristics) analysis was used. The odds ratio (OR) was estimated by logistic regression analysis to find the independent risk factors for ASB. $p<0.05$ was considered to be statistically significant.

IV. RESULTS

IV/1. Prevalence of asymptomatic bacteriuria in type 1 diabetic children

The prevalence of ASB was 10.1% (95%CI 5.7-14.5%) which was significantly higher in diabetic than in the control group [2.6% (95%CI 0.35-4.8%); (p=0.003), Figure 4.]. We did not find any difference in prevalence of ASB between diabetic males [9.3% (95%CI 3.2-15.4%)] and females [10.9% (95%CI 4.6-17.2%); (p=0.73), Figure 5.].

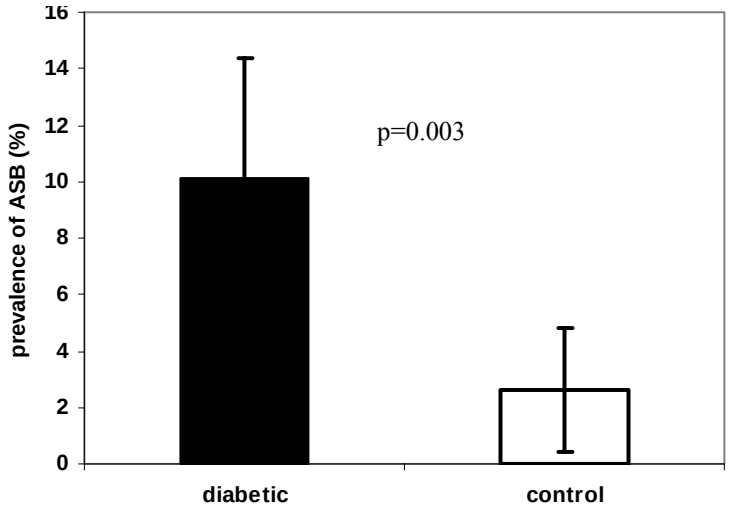

Figure 4.: The prevalence of ASB (± 95%CI) in diabetic (black bar) and control children (white bar) (p=0.003).

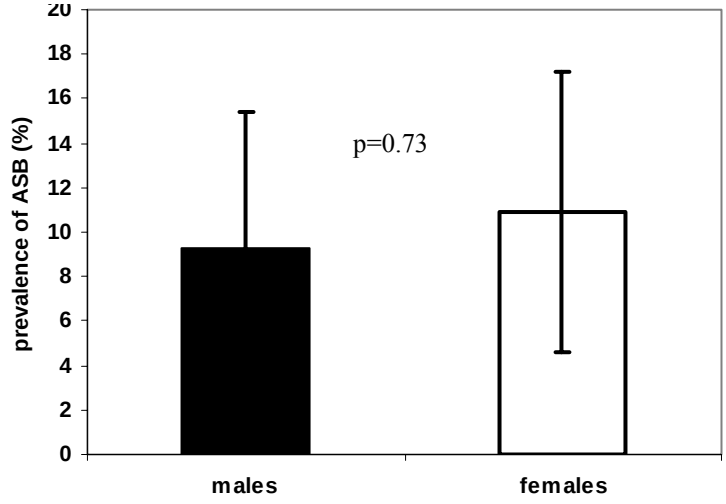

Figure 5.: The prevalence of ASB (± 95%CI) in diabetic males (black bar) and females (white bar) (p=0.73).

Dividing the participants into two groups according to age, we found that the prevalence of ASB was not significantly different in young diabetic and control patients (<15 years of age) [6.3% (95%CI 1.4-11.1%) vs. 1.7% (95%CI 0.1-2.3%); p=0.15]. In contrast, the prevalence of ASB was higher in the older diabetic subjects (≥15 years of age), than among controls [14.6% (95%CI 7.0-22.2%) vs. 3.8% (95%CI 0.2-8.0%); p=0.028)]. In this older age-group ASB was twice as common in diabetic girls than in boys but this difference was not statistically significant (data not shown). The prevalence of ASB in diabetic patients tended to increase with age ≥15 years of age [14.6% (95%CI 7.0-22.2%)] vs. <15 years of age [6.3% (95%CI 1.4-11.1%); p=0.064)]. There was no history of earlier symptomatic urinary tract infection in participants with bacteriuria.

IV/2. Possible causes of asymptomatic bacteriuria

IV/2.1. Metabolic parameters

There was no difference between diabetic patients with and without ASB in age, duration of diabetes, BMI and in pubertal development (Table 1.). HbA_{1C}, daily mean blood glucose levels, serum glucose at the time of urine sampling and daily urinary glucose excretion were also similar (Table 1.). The albumin exretion rate was somewhat higher in bacteriuric patients but it remained within the normal range (Table 1.).

Univariate logistic regression analysis did not find any significant risk factors, but HbA_{1C} ≥10% in the multivariate analysis proved to be an independent risk factor for ASB (OR: 5.23; p=0.002); (Table 2.).

Parameters	**sterile**	**ASB**	**P**
N	160	18	
Age (yrs)	14.9 ± 5.9	16.9 ± 5.1	0.17
Duration of DM (yrs) *	6.0 (2.9-9.9)	8.5 (5.1-11.9)	0.067
BMI (kg/m^2)	20.9 ± 4.4	22.0 ± 4.6	0.35
Systolic BP (mmHg)	109.6 ± 14.0	111.1 ± 10.0	0.6
Diastolic BP (mmHg)	65.7 ± 7.0	67.8 ± 7.5	0.25
Prepuberty/puberty/postpuberty (%)	23.1/37.5/39.4	16.7/22.2/61.1	0.2
Albumin excretion rate (mg/day) *	11.6 (7.4-20.2)	22.7 (9.6-28.9)	0.054
Daily mean blood glucose (mmol/day)	9.4 ± 2.1	8.6 ± 1.6	0.31
Morning serum glucose (mmol/l)	12.1 ± 4.6	11.3 ± 4.5	0.5
Urine glucose excretion (g/day) *	13.6 (5.5-26.5)	13.0 (4.4-26.8)	0.82
HbA_{1C} (%) *	8.3 (7.3-9.8)	10.0 (7.6-11.1)	0.1
Leukocyturia (%)	*14.4*	*46.7*	*0.002*

Table 1.: Comparisons between diabetic patients with and without ASB. *: median (IQR) is given because of the skewed distribution.

Variables	Univariate		Multivariate	
	p	OR (95%CI)	p	OR adjusted for age (95%CI)
Age	0.17	1.06 (0.98-1.15)	0.85	1.01 (0.89-1.15)
Gender	0.73	1.19 (0.45-3.17)	0.77	1.19 (0.37-3.87)
Duration of DM <5 yrs	0.21	1	0.26	1
5-10 yrs	0.21	2.28 (0.63-8.18)	0.27	2.65 (0.46-15.1)
>10 yrs	0.08	3.17 (0.87-11.5)	0.1	5.2 (0.72-37.1)
Microalbuminuria	0.15	2.47 (0.71-8.54)	0.5	1.58 (0.41-6.04)
HbA_{1C} >10%	0.09	2.41 (0.86-6.76)	*0.02*	*5.23 (1.29-21.3)*

Table 2.: Univariate and multivariate analysis of potential host factors associated with ASB in diabetic patients.

IV/2.2. Immunological causes – urinary interleukins

IL-6 in the urine was detectable in 39 of 178 controls [21.9% (95%CI 15.8-28.0%)] and in 24 of 133 [18.0% (95%CI 11.5-24.5%)] diabetic patients (p=0.41). The median value of IL-6 was below the detection limits both in diabetic patients and control subjects (p=0.31).

Sixty-three of 133 patients with diabetes [47.4% (95%CI 38.9-55.9%)] and only 49 of 178 of controls [27.5% (95%CI 20.9-34.1%)] had measurable IL-8 in their urine (p=0.001) and IL-8 values were significantly higher in diabetic [<3.1 pg/mg creatinine (<3.1-22.3) median (IQR)] than in control patients [<3.1 pg/mg creatinine (<3.1-4.5); p=0.001] (Figure 6.). In the diabetic group IL-8 levels were higher in girls [8.5 pg/mg

creatinine (<3.1-63.5)] as compared to boys [<3.1 pg/mg creatinine (<3.1-7.5); p=0.001].

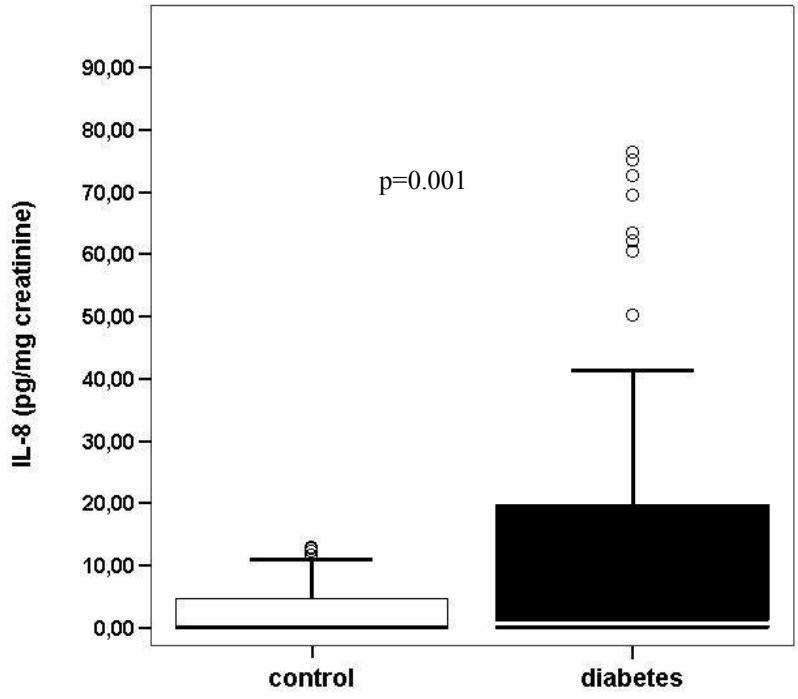

Figure 6.: Urinary IL-8 levels in control (white bar) and diabetic patients (black bar) (p=0.001). Results are presented as boxplot, the horizontal lines inside the bars represent the median, the box represents the IQR. The open circles symbolize the outlier values.

In children with ASB the IL-8 levels were similar both in the diabetic [70.0 pg/mg creatinine (<3.1-474.4)] and in the control group [42.3 pg/mg creatinine (<3.1-686.6); p=0.8], suggesting that the increased IL-8 levels in the urine were due to bacteriuria rather than diabetes itself. Indeed, urinary IL-8 concentration correlated with the level of bacteriuria, the more bacteria in the urine the higher the IL-8 value is (p=0.001); (Figure 7.).

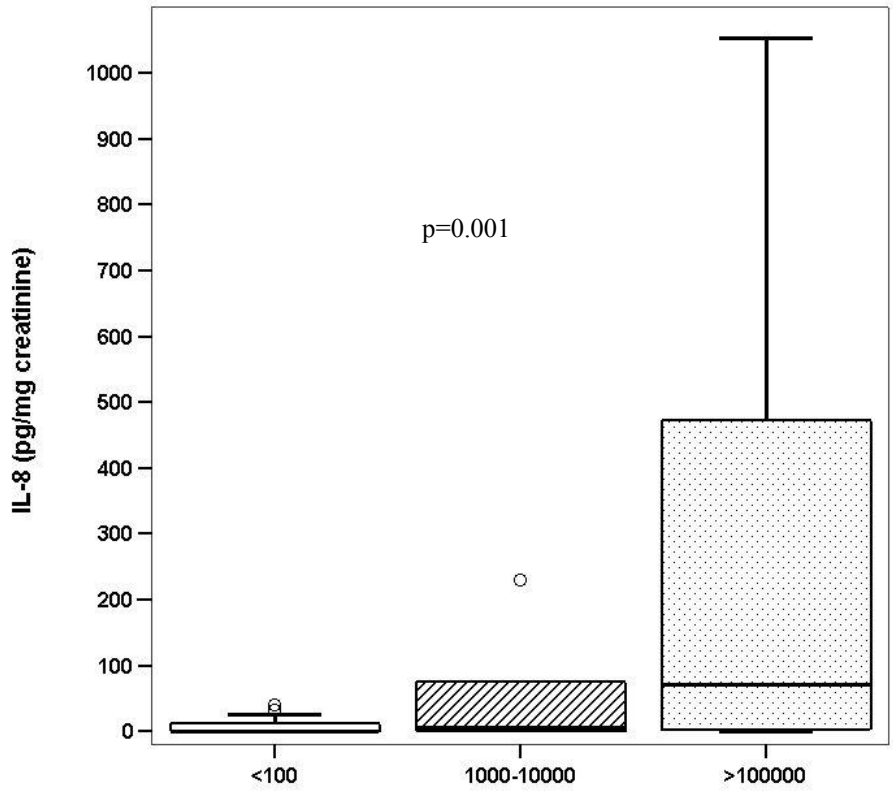

Figure 7.: Association between urinary IL-8 and the count of cultured bacteria (p=0.001, Kruskal-Wallis test). Results are presented as boxplot, the horizontal lines inside the bars represent the median, the box represents the IQR. The open circles symbolize the outlier values.

Furthermore, diabetic patients with leukocyturia had higher IL-8 concentration [20.9 pg/mg creatinine (<3.1-417.1)] than those without leukocyturia [<3.1 pg/mg creatinine (<3.1-14.3); p=0.003]. One would have expected, therefore that the prevalence of „IL-8-uria" and the urinary level of IL-8 would be low and comparable in both diabetic and control children without ASB. However, it was found that the prevalence of „IL-8-uria" was somewhat higher in diabetics without ASB [41.4% (95%CI 32.2-50.6%) as compared to controls [27.2% (95%CI 20.6-33.8%); p=0.012].

IL-8 was detectable in 77.2% (95%CI 59.8-94.6%) of diabetic patients with ASB and 41.4% (95%CI 32.2-50.6%) of them without ASB (p=0.002) and median the IL-8 level was higher in diabetics with ASB [70.0 pg/mg creatinine (2.5-474.4)] as

compared to those without ASB [<3.1 pg/mg creatinine (<3.1-12.7); p=0.001] (Figure 8.).

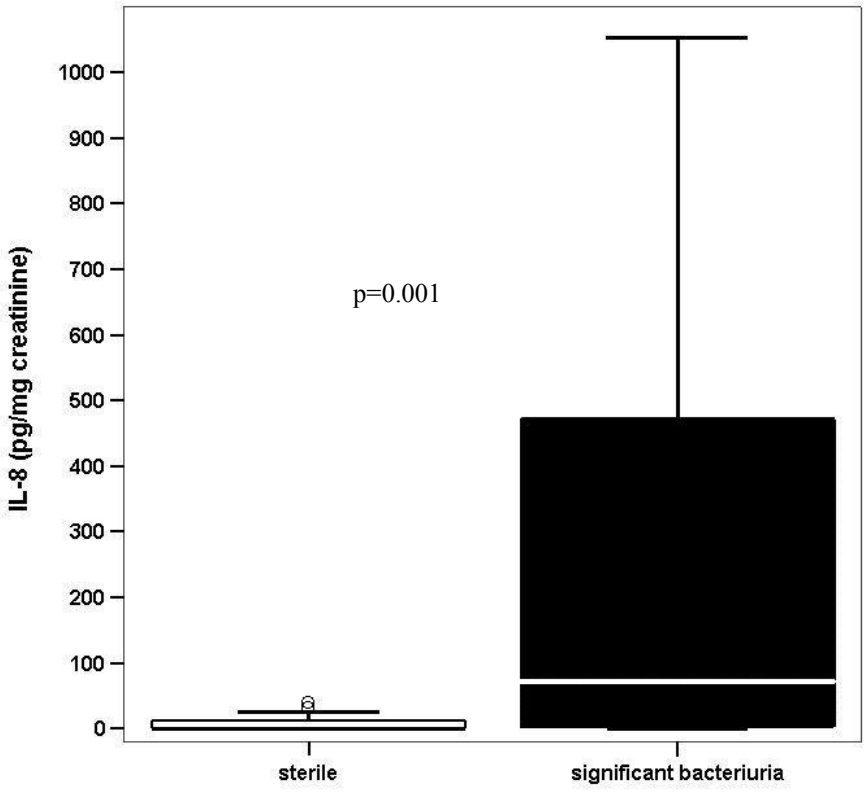

Figure 8.: IL-8 levels in diabetic patients with (black bar) and without ASB (white bar) (p=0.001). Results are presented as boxplot, the horizontal lines inside the bars represent the median, the box represents the IQR. The open circles symbolize the outlier values.

In the diabetic group a weak positive correlation was observed between HbA_{1C} and detectable IL-8 levels (≥3.1 pg/mg creatinine) (r=0.4; p=0.002), but similar association was absent between HbA_{1C} and detectable IL-6 levels (≥4.7 pg/mg creatinine) (r=-0.1; p=0.43).

In the diabetic group there was no difference in IL-8 levels between patients with gram-negative [134.6 pg/mg creatinine (5.8-417.1)] and gram-positive bacteriuria [70.0 pg/mg creatinine (<3.1-535.3)] (p=0.76).

IV/2.3. Expression of CXC-receptor-1

The expression of CXCR-1 on the surface of neutrophils in type 1 diabetic children was significantly lower than that in control group [240.4 (95%CI: 232.2-248.7) vs. 256.1 (95%CI: 246.3-266.0); p=0.024] (Figure 9.). There was no difference between males and females either in the diabetic [237.5 (95%CI: 224.7-250.3) vs. 242.2 (95%CI: 231.3-253.4); p= 0.57] or in the control group [259.9 (95%CI: 243.7-276.2) vs. 253.5 (95%CI: 240.4-266.5); p= 0.52]. CXCR-1 expression in the diabetic group correlated with age (r=0.22; p=0.019), duration of diabetes (r=0.21; p=0.025), but not with BMI (r=0.01; p=0.99), HbA_{1C} (r=-0.068; p=0.48) and albumin excretion rate (r=0.1; p=0.29).

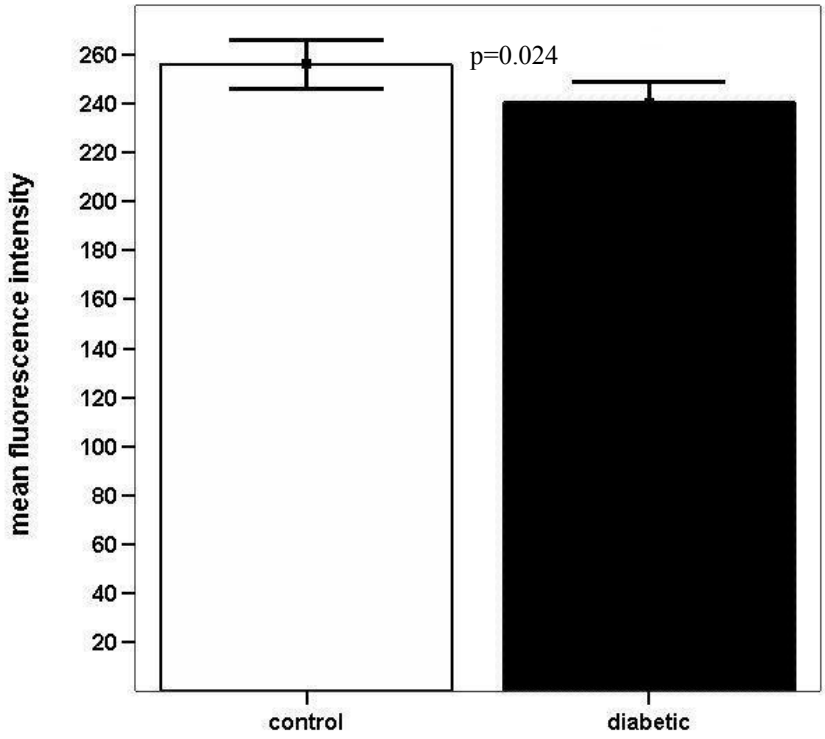

Figure 9.: The mean fluorescence intensity and 95%CI of CXCR-1 are represented in control (white bar) and in diabetic groups (black bar) (Student's t test; p=0.024).

IV/3. Serum level of interleukin-8 in type 1 diabetic children

Serum IL-8 was detectable in 46.8% (95%CI 35.8-57.8%) of diabetic and 30.0% (95%CI 15.8-44.2%) of healthy children (p=0.07). However, the concentration of IL-8 was higher in diabetic children [< 5 pg/ml (< 5-6.4 pg/ml)] than in control group [< 5 pg/ml (< 5-5.2 pg/ml); p=0.025] (Figure 10.). In the diabetic patients there was no difference between age, BMI, HbA_{1C}, cholesterol or triglyceride levels and microalbuminuria in the subgroups with detectable and non-detectable IL-8 levels (Table 3.). The only significant difference was observed in the duration of diabetes. Diabetic patients with detectable IL-8 level had longer diabetes duration (9.2 ± 5.3 years), than those without detectable IL-8 levels (6.5 ± 4.3 years; p=0.018).

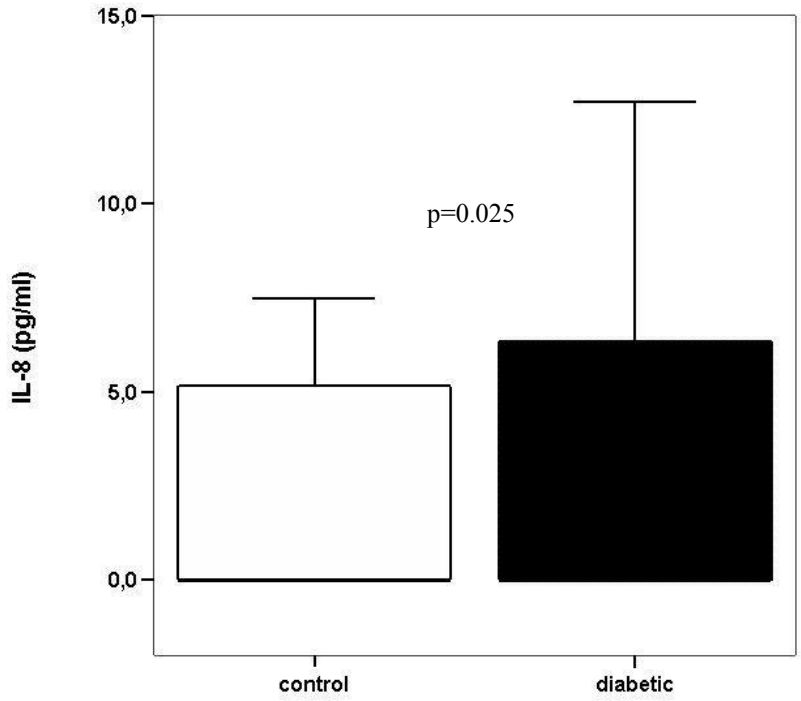

Figure 10.: The serum IL-8 concentrations in control (white bar) and diabetic children (black bar) are represented as a boxplot. The box shows the interquartile range (25-75%) and the whisker cap spreads to 90%. The median line and the 25% line overlap. (Mann-Whitney test, p=0.025).

IL-8	Detectable	Not detectable	p
Prevalence (%)	46.8	53.2	
Age (years)	15.8 ± 4.4	14.8 ± 4.9	0.38
Diabetes duration (years)	*9.2 ±5.3*	*6.5 ±4.3*	*0.018*
HbA_{1C} (%)	8.2 ± 2.0	8.1 ± 1.6	0.66
Cholesterol (mM/l)	4.2 ± 0.8	4.4 ± 0.9	0.55
Triglyceride (mM/l)	1.0 ± 0.7	1.0 ± 0.4	0.84
BMI (kg/m^2)	22.1 ± 4.4	21.9 ± 5.3	0.88
Microalbuminuria (%)	18.9	30.9	0.22

Table 3.: Parameters of type 1 diabetic children with detectable and undetectable IL-8 levels. The prevalence of detectable IL-8 and microalbuminuria are given as percent, the other variables are presented as mean ± SD.

Logistic regression analysis revealed that duration of diabetes (OR: 1.16; p=0.023) is a significant risk factors of appearance of IL-8 in the serum of patients with type 1 diabetes (Table 4.).

Risk factors	OR (95%CI)	p
Age	0.95 (0,85-1.1)	0.48
Diabetes duration	*1.16 (1.02-1.3)*	*0.023*
HbA_{1C} (≥8%)	0.62 (0.24-1.62)	0.33
BMI	1.03 (0.89-1.19)	0.68
Microalbuminuria	0.44 (0.13-1.52)	0.19

Table 4.: Risk factors of IL-8 in the serum of type 1 diabetes.

IV/4. Bacterial specimens and evaluation of pyuria

Bacteria cultured in the diabetic group are listed in Table 5. Gram positive and Gram negative bacteria were found in 11 and 7 cases, respectively, and the proportion of leukocyturia in patients with Gram positive and Gram negative bacteria was 2/11 to 6/7 (p=0.041), respectively (Table 4.). The frequency of E. coli which is a dominant pathogen agent in UTI was low in diabetic children with ASB (in four out of 14 cases). In controls, E. coli (n=2), Streptococcus agalactiae, Klebsiella pneumoniae and Proteus vulgaris (n=1-1) were cultured. Among bacteriuric controls only one patient with Proteus vulgaris had leukocyturia.

Bacteria	**Gram-staining**	**Case/Σn**	**Leukocyturia/cases**
S. agalactiae	positive	6/18	2/6
Enterococcus sp.	positive	5/18	0/5
E. coli	negative	4/18	4/4
K. pneumoniae	negative	3/18	2/3

Table 5.: Frequency of isolated bacteria and leukocyturia among diabetic patients with ASB.

The frequency of leukocyturia was 17.9% (95%CI 11.5-24.3%) in diabetic patients and 7.9% (95%CI 4.1-11.7%) in controls (p=0.006). Leukocyturia without ASB also tended to be more frequent in diabetic patients than in controls [14.4% (95%CI 8.2-20.5%) vs. 7.5% (95%CI 6.4-8.6%); p=0.05]. In the diabetic group, leukocyturia was more frequent in patients with ASB [46.7% (95%CI 21.5-71.9%)] as compared to those without ASB [14.4% (95%CI 8.2-20.5%); p=0.002] (Table 1.).

IV/5. Usefulness of leukocyturia and urinary IL-8 in screening for ASB

In the whole population the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of leukocyturia - to diagnose ASB - were showed in Table 6. We estimated the optimal cut-off point of urinary IL-8 level for the prediction of ASB by ROC analysis (Figure 11.). Using this method the optimal cut-off point of was an IL-8 level of 10.2 pg/mg creatinine. If this value would be used for screening, the sensitivity, specificity, PPV and NPV were presented in Table 6.

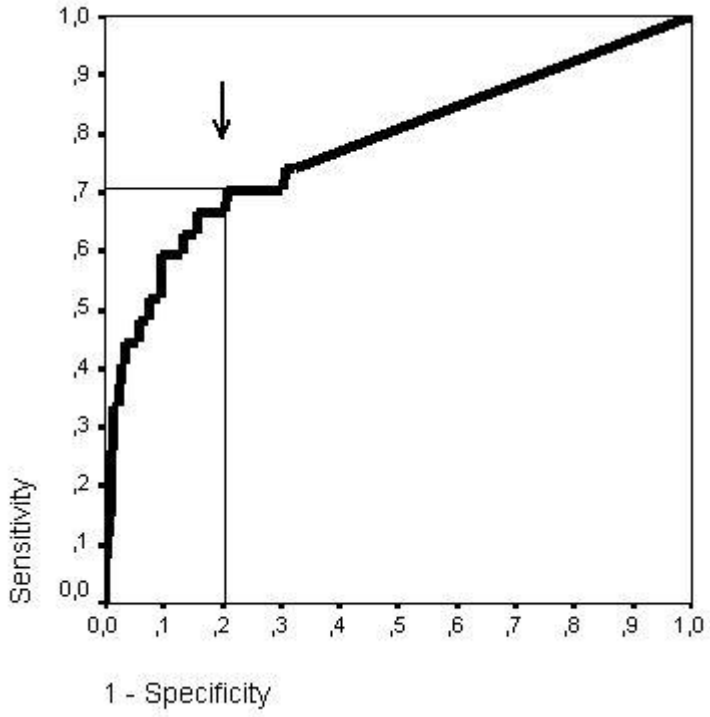

Figure 11.: Analysis of the optimal cut-off point of IL-8 for screening ASB by ROC analysis. Arrow shows the value of 10.2 pg/mg creatinine IL-8, the narrow lines are interpolated to the x and y axes to show the sensitivity (70.4%) and the specificity (78.9%).

	leukocyturia	IL-8	leukocyturia+IL-8
Sensitivity (%)	50.0	70.4	81.5
Specificity (%)	89.9	78.9	71.8
PPV (%)	31.7	23.8	21.6
NPV (%)	95.1	96.5	97.6

Table 6.: The effectiveness of screening methods of ASB. In the case of IL-8 the value of 10.2 pg/mg creatinine (calculated by ROC analysis) was used as cut-off point.

The sensitivity of „IL-8-uria" is higher than leukocyturia for screening of ASB, but the PPV of leukocyturia is better. The sensitivity can be enhanced using leukocyturia and „IL-8-uria" together, but the same time specificity would be diminished (Table 6.).

V. DISCUSSION

V/1. Prevalence of ASB

As described by Geerlings et al. (40) one out of five type 1 diabetic women had ASB. In the few studies in diabetic children a low prevalence of about 1% was found (42-44). Our clinical experience suggested a much higher prevalence, therefore we decided to estimate the prevalence and the possible risk factors of ASB in type 1 diabetic children. In contrast to studies mentioned above we have found that the prevalence of ASB was four-fold higher in diabetic children and young adults as compared controls (Figure 4.). It seems unlikely that this was due to contamination because the sampling was carried out in the same manner in both diabetic and control group and we excluded cultures in which more than one species was grown. One possible explanation for the discrepancy between the published prevalence and the one we have found could be that urine sampling was different in these studies (42-44). In these publications, there was an interval of 5 to 14 days between the two urine samplings, and after 7-10 days of the first culture the second samplings were negative in about one third of the patients (45). Whereas in our study urine was collected on two consecutive mornings.

The prevalence was higher in both diabetic boys and girls, we could not observe the gender difference of ASB (female preponderance), a phenomenon frequency seen in diabetic adults (39). However, above 15 years of age the prevalence of ASB was higher in diabetic girls, than in boys, but this difference was not statistically significant probably because of the fewer count of subjects.

It has been also described that the prevalence of ASB increases with age (35, 39). The prevalence in our study was not significantly different in patients under and above of 15 years of age, but it tended to increase with age.

Several studies in adult diabetic women have shown that the glycemic control did not influence the prevalence of ASB (39, 40, 45, 46). Our observation suggests that higher HbA_{1C} levels tend to promote ASB. Comparing diabetic patients with and without ASB we did not find differences in HbA_{1C} levels, but using multiple regression analysis a HbA_{1C} levels above 10% (equivalent to the 75 percentile in this study) did increase the risk of the development of ASB (Table 2.).

It was suggested that peripheral neuropathy, retinopathy and macroalbuminuria are significant risk factors for ASB in adult type 1 diabetic women (39, 40, 46, 50). All three of these conditions are rare in childhood diabetes because of the relatively short duration of diabetes. The albumin excretion rate tended to be elevated in diabetic patients with ASB, but the median and IQR of AER was in the normal range in both groups (Table 1.). It is possible that the tendency to elevated albumin excretion was due to the bacteriuria in our patients. It is known that even a mild infection in the urinary tract can cause transient elevation in urinary albumin excretion, therefore at the evaluation of microalbuminuria the presence of bacteriuria has also to be taken into account (51).

V/2. Urinary interleukins

Recently, the attention has turned to the features of host defense mechanisms of patients with ASB. Cytokines play a major role in mediating the inflammatory process in various clinical entities. IL-6 as a heat-shock protein and IL-8 as the main chemoattractant have been found in increased concentrations in the urine of non-

diabetic patients with UTI (including ASB) not only in adults (13, 19), but also in children (16, 18).

However, in a recent study, lower proinflammatory cytokine secretion (IL-6 and IL-8) and urinary leukocyte cell count were found in the urine of adult diabetic women with ASB compared to healthy women with ASB (62). An impaired host defense mechanisms – suggested by these findings - were thought to play an important role in the higher susceptibility to ASB in diabetic patients.

IL-6 was detectable in the urine of only one fifth of both diabetic and control children in our study and the median value was similarly below the detection limit in both groups. In diabetic children with ASB urinary IL-6 concentration was similar to those of non-diabetic controls.

However, the IL-8 response was more frequent in diabetic patients than in controls and the median value of IL-8 was also higher in the diabetic subjects (Figure 6.). Diabetic patients and controls with ASB, however, had similar IL-8 levels suggesting that the host defense mechanisms associated with urinary interleukins was not diminished in type 1 diabetic children and young adults.

Interestingly, we have also found detectable IL-8 values in individuals (both diabetic and control patients) without ASB. There are several possible explanations for this phenomenon. Hang et al. have observed that the epithelial cells of the urinary tract had significant IL-8 staining by monoclonal antibodies but in contrast to the significant marking of the cells, IL-8 was not always measurable in the urine. According to their *in vitro* experiments the IL-8 production by the epithelium seemed to be constitutive and the secretion can be induced by different noxious agents entering the urinary tract (15). Another possible explanation of this finding could simply be the definition of „sterile" urine: bacterial count less than 10^5 CFU/ml. In other words, individuals labeled

„without ABS" based on this definition could still have bacterial counts which were low but sufficient enough to produce some IL-8 production. Furthermore, in patients with contaminated cultures the urinary IL-8 level was higher than in „no-bacteriuria" group and was in the range similar to that in ASB group (data not shown).

V/3. Leukocyte CXCR-1 expression and serum IL-8 concentrations

We have demonstrated a decreased CXCR-1 expression on the neutrophils of children and young adults with type 1 diabetes as compared to healthy controls. It seemed that the expression of CXCR-1 was not influenced by metabolic parameters, including HbA_{1C}, but correlated with age and duration of diabetes. Regarding the age-dependency of CXCR-1 expression in type 1 diabetes, it is important to note, that if the confounding effect of diabetes duration was excluded using partial correlation analysis, the correlation between age and CXCR-1 expression disappeared. This correlation was not observed in the control group (data not shown). Therefore, it seems that not age per se, but diabetes duration could influence the CXCR-1 expression.

Frendéus et al. examined the neutrophils of children with recurrent pyelonephritis in an infection-free period and demonstrated a decreased CXCR-1 expression (29). The decreased CXCR-1 expression could lead to diminished neutrophil function and could be one possible factor in the susceptibility to infections in diabetes.

In an attempt to find a possible explanation for the lower CXCR-1 expression in diabetes we studied the serum concentration of the selective ligand of CXCR-1, namely IL-8. In agreement with a recent study, higher IL-8 level was found in diabetic children than healthy controls (63, 64). We have also observed an association between diabetes duration and IL-8 levels, supporting the hypothesis, that there is a low grade

inflammation in diabetic patients even in the absence of micro- or macrovascular complications (65).

The effect of hyperglycaemia on IL-8 secretion was investigated in the *in vitro* study of Temaru et al. (66). They have shown an enhanced IL-8 messenger RNA production by human aortic endothelial cells, but not by smooth muscle cells as a response to increasing glucose concentrations. In our study, HbA_{1C} did not seem to influence the serum IL-8 levels in diabetes. However, short hyperglycaemic periods - which are often found in type 1 diabetic children and not necessarily reflected in HbA_{1C} levels - may have contributed to the elevated IL-8 concentration.

Furthermore there is also known that diabetic ketoacidosis causes elevation of serum level of many cytokines including IL-8. The increase of IL-8 concentration was pronounced after 6-8 hours of the initial treatment and normalized at the 5th day of therapy. The authors considered this observation as a part of a hyperinflammatory response accompanying diabetic ketoacidosis (67). Our observations were made in diabetic patients without ketoacidosis, but we measured the IL-8 level of few subjects with ketoacidosis and found similar tendency of IL-8 kinetics cited above (unpublished observation).

The significance of the lower CXCR-1 expression in type 1 diabetic children is not yet known. Neutrophils of healthy subjects stimulated by bacterial antigens *in vitro*, especially by lipopolysaccharide (LPS) have shown a quick and sustained down-modulation of CXC-receptors within a few minutes after adding the stimulant (68, 69). The down-regulation of CXC-receptors were studied in many pathological circumstances. In sepsis (25), inflammatory arthritis (70), asthma (26) and Helicobacter infection (27) the down-modulation of CXC-receptors were more prominent than in control group contributing to the depressed host defense mechanisms. There are no data

available on the kinetics of CXC-receptors expression in diabetic subjects after stimuli by different pathogenic agents.

The measurements of CXCR-1 expression and IL-8 level were performed in different cohorts of diabetic and non-diabetic individuals, therefore the interpretations of the results have limitations. However, the sex ratio, the age, the duration of diabetes and the glycated Hb level were similar in the different cohorts.

V/4.Types of bacteria in the urine and lekocyturia

In UTI the most common bacterium is usually E. coli, which was isolated in about 80-90% of positive cultures (1, 4). In our diabetic patients with ASB E. coli was found in only about a quarter of positive cases (Table 3.). Similar rates were found in adult type 1 diabetic women with ASB (40). It seems therefore, that the spectrum of pathogenic bacteria causing ASB and UTI is different. This may be due to the different virulence factors in ASB and UTI. E. coli strains causing ASB had fewer virulence factors than those causing UTI. P fimbriae - the main virulence factor of E. coli being responsible to the adherence of the bacteria to the uroepithelium - were expressed in 46% of specimens isolated from patients with UTI, but were not detectable in strains in patients with ASB. These strains were mainly not able to adhere to the uroepithelium, at all (71).

According to our observation gram-negative bacteria were present in only 38.8% and gram-positive species in 61.2% of the cases. The IL-8 levels were similar in urines with gram-negative and positive bacteria also suggesting that gram-positive bacteria (S. agalactiae, Enterococcus sp.) in the urine of a diabetic patient can induce secretion of interleukines by stimulation of the uroepithelial cells. Therefore, we can assume that gram-positive bacteria can also be pathogenic in the urinary tract.

The frequency of leukocyturia was also higher in diabetic children than in controls. Nearly half of our bacteriuric diabetic patients had leukocyturia. A similar finding was described in adult diabetic women (40, 72). To estimate the sensitivity, specificity, PPV and NPV of leukocyturia we combined the microscopic findings and the dip-slide test (leukocyte-esterase). In accordance with others using leukocyte-esterase test (72, 73) a sensitivity of 50.0% and a PPV of 31.7% was reached. A sensitivity and PPV of this magnitude is not sufficient to reliably identify diabetic patients with ASB. Therefore we also examined the urinary IL-8 as a possible predictor of ASB in diabetes. By means of ROC analysis we have reached a much better sensitivity (70.4%) for IL-8, but similar PPV as in the case of leukocyturia, similarly to other studies (73). The cause of the lower PPV could be explained by its earlier release from the site of infection, earlier disappearing from the urine than PMNs or bacteria or inflammation elsewhere in the body (15).

The association between the number of polymorphonuclear leukocyte and the urinary IL-8 is well known (11, 13). In a recent study, a lower urinary leukocytes cell count was found in diabetic women and therefore a moderate immune responses were presumed in diabetes explaining the higher susceptibility to urinary tract infections (62). We did not have the possibility to count the exact number of PMNs in the urine, only the leukocyturia was recorded. We found higher IL-8 level in individuals with leukocyturia which shows an intact „IL-8-PMNs" axis in the urinary tract of a type 1 diabetic children.

VI. SUMMARY OF NEW OBSERVATIONS

▪ We have found that the prevalence of ASB is four-fold higher in diabetic children and young adults than in controls. Gender difference is not remarkable. Poor glycemic control (HbA_{1C} ≥10%) appears to be a significant risk factor.

▪ There is no impairment in urinary cytokine network (including IL-6 and IL-8) in diabetic children with ASB.

▪ Low grade inflammatory state – presented by elevated serum IL-8 concentration - in type 1 diabetes may cause down-regulation of CXCR-1, therefore it could contribute to the enhanced susceptibility to several infections in diabetic patients. Further investigations are needed to reveal the kinetics of CXCR-1 expression and understand the clinical importance of this observation.

▪ The spectrum of pathogenic bacteria in ASB differs from the usual spectrum of UTI, not only gram negative but gram positive strains are also present.

▪ Urinary IL-8 together with determination of leukocyturia had good sensitivity to screen ASB, unfortunately the positive predictive values of these methods are low.

VII.ABBREVIATIONS

AER: albumin exretion rate

ASB: asymptomatic bacteriuria

CFU: colony-forming units

CXCR-1: CXC-receptor-1

HbA_{1C}: hemoglobin A_{1C}

hpf: high-power field

IL-6: interleukin-6

IL-8: interleukin-8

LPS: lipopolysaccharide

NPV: negative predictive value

PMNs: polymorphonuclear leukocytes

PPV: positive predictive value

ROC: receiver operating charecteristics

RR: relative risk

THP: Tamm-Horsfall protein

TLRs: Toll-like receptors

UTI: urinary tract infection

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X. PUBLICATIONS AND LECTURES

Publications, abstracts, lectures and posters on which the thesis was based

Publications

• Rózsai B., Lányi É., Soltész Gy: Asymptomatic bacteriuria and leukocyturia in type 1 diabetic children and young adults. *Diab Care* 26: 2209-2210, 2003. **IF: 7.5**

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