Pathogenesis, Diagnosis, and Consequences of Bacteriuria In Women with or without Diabetes Mellitus

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ISBN-10: 90-9020674-4
ISBN-13: 978-90-9020674-5

Lay-out: R. Sanders
Printing: Febodruk B.V.

Dit proefschrift is tot stand gekomen door financiële ondersteuning van: Stichting "De Drie Lichten", Vakgroep Acute Geneeskunde en Infectieziekten van het UMC Utrecht, AstraZeneca B.V. , Janssen-Cilag B.V.

Pathogenesis, Diagnosis, and Consequences of Bacteriuria In Women with or without Diabetes Mellitus

Pathogenese, diagnostiek en gevolgen van bacteriurie bij vrouwen met of zonder diabetes mellitus

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op
vrijdag 2 juni 2006 des middags te 12.45 uur

door

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geboren op 7 januari 1973, te Zaandam

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Aan Eric en Pepijn

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One

General introduction

Translated from the Greek, the word diabetes means siphon, referring to the uncontrolled flow and loss of urine in patients with hyperglycemia ('dia'= trough, 'bainoo'= to go). The term was first used by Aretaeus, a disciple of Hippocrates, who described diabetes as "a wonderful affection, not very common among men, being a melting down of the flesh and limbs into urine...". The Latin mellitus, meaning honey-sweet, was added because the ancient Hindus recognized the sweet taste of urine in diabetic patients.[1]
Although the polyuria of diabetic patients with hyperglycemia is not related to the pollakisuria caused by urinary tract infections (UTIs), a clear association between UTIs and diabetes mellitus (DM) does exist.

Diabetes mellitus is a metabolic disorder, characterized by an increased blood glucose level, resulting from a defect in insulin secretion, resistance to insulin action, or both. Although perhaps not very common in ancient times, nowadays its prevalence is increasing drastically. The prevalence of DM among adults worldwide was estimated to be 4.6% in 2000 and 6.4% in 2030 (in the Netherlands 3.5% and 5.4%, respectively).[2] The prevalence increases with age. Two population-based studies performed in the Netherlands reported a prevalence of 8.3% in elderly aged 50 to 74 years, and 10.9% in women aged 55 years and older, respectively.[3,4] Besides organ complications as retinopathy, nephropathy, neuropathy and cardiovascular diseases, patients with DM also more frequently experience (complicated) infections compared with patients without diabetes. The urinary tract is the most prevalent site of infection.[5]

Urinary tract infections are common, especially among women and elderly. In the Dutch general practitioner's office the mean incidence of an acute cystitis is 70 per 1000 women per year, increasing to almost 200 per 1000 per year among women aged 75 years.[6] In general, an uncomplicated UTI is defined as a community-acquired lower UTI in an otherwise healthy nonpregnant adult woman without urinary tract abnormalities.[7] This would mean that all UTIs occurring in women with DM should be considered complicated. Women with DM are more susceptible to both asymptomatic and symptomatic UTIs.[8,9] For instance, most studies are consistent in finding a 2- to 4-fold higher prevalence of asymptomatic bacteriuria (ASB) in women with DM compared to women without DM.[8,10] In men no increased prevalence has been documented. Besides the higher incidence, UTIs in diabetic patients are also more likely to have a complicated course, sometimes leading to serious complications as sepsis or renal abscesses.[11] This suggests a difference in pathogenesis between diabetic and nondiabetic patients. Several possible mechanisms have been studied before and are reviewed in **Chapter 2**. The presence of bacteriuria without accompanying symptoms of a UTI is called ASB. In nondiabetic patients ASB has been associated with the development of hypertension, renal impairment and with increased mortality.[12] A causal relationship however has never been established. More recently, the results of a prospective study of our study group showed that in women with DM type 2, ASB is a risk factor

for the development of a symptomatic UTI. Moreover, women with DM type 1 and ASB showed a tendency to a faster decline in renal function than those without ASB (relative increase in creatinine 4.6% versus 1.5% after 18 month follow-up, p= 0.2).[13] Considering the above, it would seem prudent to screen all diabetic females on ASB and, if present, to treat them with antimicrobial therapy. However, it has never been shown that ASB in itself can lead to severe complications, such as renal function deterioration. Therefore, we decided to study the possible long-term consequences of ASB on renal function and the development of hypertension. If it is true that ASB has severe consequences, it is important to define the best screening strategy as well as the best therapeutic management to find out if the consequences can be prevented.

Objectives and build-up of this thesis

Part one: Pathogenesis and Diagnosis

In **Chapter 2** a more detailed overview is given of the current literature on UTIs in women with DM, with an emphasis on epidemiology, pathogenesis and management.

The first step in the pathogenesis of UTIs is the adherence of the uropathogen to the epithelium that is lining the bladder surface. We hypothesized that the increased prevalence of bacteriuria in women with versus those without DM is based on an increased adherence of *Escherichia coli* (the most prevalent uropathogen) to bladder cells from women with DM (**Chapter 3**).

The adherence of *E. coli* is mediated by the FimH adhesin at the tip of type 1 fimbriae. FimH antiserum inhibits the adherence of *E. coli* to bladder cells of nondiabetic women.[14] Hypothesizing that an increased adherence to cells from diabetic women might be due to a difference in uroepithelial cell receptor, we studied whether FimH antiserum can also inhibit the adherence of *E. coli* to diabetic cells (**Chapter 4**). When a difference is present this could have consequences for patients with DM for the current studies on antibacterial approaches, such as vaccines.

As sometimes suggested by others,[15] we wanted to answer the question whether DM in itself is a risk factor for higher antibiotic resistance in uropathogenic *E. coli* (**Chapter 5**).

Chapters 6 and 7 concern the diagnosis of UTIs. We tried to find alternatives for the classical urine culture to diagnose bacteriuria. First, we studied whether in diabetic women ASB can be diagnosed by history taking alone, and the added value of leukocyturia. In addition, we developed a real-time Polymerase Chain Reaction (PCR) to detect *E. coli*-bacteriuria.

Part two: Consequences

Although ASB is a very common phenomenon that can remain present for a long period, there is still no evidence that it can lead to serious complications in women without or women with DM. Therefore, we studied whether ASB is associated with a faster decline in renal function, a higher incidence of end-stage renal failure, or with the development of hypertension in a generally healthy population of adult women (**Chapters 8 and 9**) and, after a motivation in **Chapter 10**, also in a population of women with DM (type 1 or type 2; **Chapter 11**).

Finally, the findings of the above mentioned studies are summarized and discussed in **Chapter 12**.

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Two

Management of bacterial urinary tract infections in adult patients with diabetes mellitus

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Drugs 2002;62(13):1859-68

Abstract

Urinary tract infections (UTIs) are more common and tend to have a more complicated course in patients with diabetes mellitus (DM) than in the general population. The mechanisms that potentially contribute to the increased prevalence of both asymptomatic and symptomatic bacteriuria in these patients are defects in the local urinary cytokine secretions and an increased adherence of the microorganisms to the uroepithelial cells. The need for treatment of asymptomatic bacteriuria (ASB) remains controversial. No evidence is available on the optimal treatment of acute cystitis and pyelonephritis in patients with DM. Because of the frequent (asymptomatic) upper tract involvement and the possible serious complications, many experts recommend a 7- to 14-day oral antibacterial regimen for bacterial cystitis in these patients, with an antibacterial agent that achieves high concentrations both in the urine and in urinary tract tissues. The recommended treatment of acute pyelonephritis does not differ from that in patients without DM. Clinical trials specifically dealing with the treatment of UTIs in patients with DM, comparing the optimal duration and choice of antibacterial agent, are needed. In addition, new approaches to preventive strategies must prove their value in this specific patient group.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections.[1] Up to 50% of women report having had at least one UTI in their lifetime.[2] Uncomplicated UTIs occur most often in young healthy adult women and are easy to treat. However, in other patient groups, UTIs can have a complicated course, are more difficult to treat, and often recur. Complicated UTIs occur most commonly in patients with abnormalities of the genitourinary tract. However, other subtle conditions such as age over 65 years, treatment with immunosuppressive drugs, the presence of Human Immunodeficiency Virus (HIV)-infection and last, but not least, diabetes mellitus (DM) also predispose to an enhanced susceptibility for the development of a UTI with a complicated course.[3,4]

DM is the most common endocrine disease. Besides organ complications as retinopathy, nephropathy and neuropathy, diabetic patients also more frequently experience (complicated) infections compared to nondiabetic patients. In a large study of patients with bacteraemia, it was demonstrated that two thirds of the patients had DM; the urinary tract was the most prevalent infection site.[5] In this chapter we focus on UTIs, although we are aware that infections elsewhere are also very important, particularly in men with DM. Furthermore, it is important to realize that most of the research described here, has been performed in female patients who have a higher prevalence of UTIs than men. Firstly this chapter briefly describes specific aspects of the epidemiology, pathogenesis, clinical presentation and consequences of asymptomatic and symptomatic UTIs in adult patients with DM, followed by a more extensive description of the management of bacteriuria in these patients. Because of the specialized character, the treatment of the complications of UTIs will not be described.

1. Epidemiology

The majority of the infections in diabetic patients is localized in the urinary tract.[5] An autopsy study in 1940 showed that approximately 20% of patients with DM had a serious infection of the urinary tract. The authors stated that this prevalence was five times higher than found in studies with nondiabetic patients.[6] Although different studies show a wide range, nearly all investigators report that the prevalence of asymptomatic bacteriuria (ASB) in women with DM is two to four times higher than in women without DM.[7,8] In men, results are more consistent with a frequency between 1 to 2% found, and no difference between men with DM and those without.[9] The frequency of symptomatic infections in women with DM is also increased.[10]

Both men and women with DM have an increased risk of acute pyelonephritis requiring hospital admission. In a recent study, diabetes was estimated to increase the probability 20- to 30-fold under the age of 44 years, and 3- to 5-fold over the age of 44 years.[11] Furthermore, complications of an upper UTI are more likely to occur in diabetic patients. For example, emphysematous pyelonephritis is seen almost exclusively in diabetic patients and, although uncommon, half the patients with papillary necrosis have diabetes.[12]

2. Pathogenesis of urinary tract infections

2.1 General

UTIs almost exclusively arise from the ascending route. Bacteria colonizing the perineum and vagina can enter the bladder and further ascend to the kidneys. The most important defense mechanisms of the host are the urine flow from the kidneys to the bladder and intermittent voiding resulting in complete emptying of the bladder. Patients with urinary obstruction, stasis and reflux have more difficulty in clearing bacteria and these conditions also seem to predispose to the development of a UTI, although exact data are lacking.[13]

The essential step in the pathogenesis of UTIs is the adherence of uropathogens to the bladder mucosa. Adhesins (fimbriae) are therefore important virulence factors. Although virulence factors have been characterized best in *Escherichia coli* (the most common uropathogen), many of the same principles may be applicable to other Gram negative uropathogens, for example *Klebsiella* spp.[14] Type 1 fimbriae mediate the adherence of *E. coli* to glycoprotein receptors (uroplakins) on the uroepithelial cells, whereas P fimbriae bind to glycolipid receptors in the kidney.[15]

2.2 Patients with diabetes mellitus

The increased frequency of UTIs in patients with DM is most likely because of several factors (*Table 1*). Suggested host-related mechanisms are a) the presence of glycosuria; b) defects in neutrophil function; and c) increased adherence to uroepithelial cells. Our in vitro studies indeed showed that glycosuria enhances the growth of different *E. coli* strains,[16] however, this was not confirmed by *in vivo* studies, which failed to show a higher prevalence of bacteriuria among diabetic patients with glycosuria than in patients without glycosuria.[8,17]

The data on impaired neutrophil function are contradictory.[22,23] Moreover, the incidence of UTIs is not increased in other groups of patients with neutrophil defects or neutropenia.[24] Local cytokine secretion might be of importance. Cytokines are small proteins that play an important role in the regulation of host defences against systemic and local bacterial infections.[25] Therefore, we investigated urinary cytokine excretion in diabetic patients and found lower urinary IL-8 and IL-6 concentrations ($p=0.1$ and $p<0.001$, respectively) in women with DM than in nondiabetic controls. A lower urinary leukocyte cell count correlated with lower urinary IL-8 and IL-6 concentrations ($p<0.05$).[18] This might contribute to the increased incidence of UTIs in this patient group.

Most interestingly, we have found that the adherence of type 1-fimbriated *E. coli* to uroepithelial cells of women with DM is increased, compared to the adherence to uroepithelial cell of women without DM (*see Chapter 3*).[19] Thus, it seems that this increased adherence plays an important role in the pathogenesis of UTIs in women with DM.

As part of the immune response, infection and adherence of the bacteria to

Table 1 Host factors associated with an increased risk for symptomatic or asymptomatic urinary tract infections (UTIs) in women with diabetes mellitus (DM)

General:
sexual intercourse[17]
history of (recurrent) UTIs[8]
obstruction, urine stasis, reflux, instrumentation of urinary tract[13 a]
Associated with (complications of) DM:
peripheral neuropathy[8]
macroalbuminuria[8]
longer duration of DM[8]
glycosuria (*in vitro*)[16]
decreased urinary cytokine secretion[18]
increased adherence of *E. coli* to uroepithelial cells[19]
Genetic factors[a]:
secretor status[20]
blood group[20]
history of UTIs of the mother[21]

[a] Not studied specifically in patients with DM

uroepithelial cells stimulates cytokine and chemokine secretion, as well as exfoliation of the superficial cells. It has been thought for a long time that uropathogenic *E. coli* are noninvasive pathogens. However, a recent study in mice has shown that type 1-fimbriated *E. coli* can not only lead to exfoliation, but can also invade the uroepithelial cells, replicate and form quiescent intracellular reservoirs that can serve as a possible source for recurrent UTIs.[26] Because we found lower urinary cytokine concentrations in women with DM,[18] we hypothesized that in these patients bacteria might invade uroepithelial cells more easily and, by an impaired inflammatory response, evade the innate host defenses.[15] This would explain why relapses of UTIs occur often in these patients.[27] Future studies will have to provide the evidence for this phenomenon.

2.3 Associated risk factors

Factors that have been proposed to constitute an enhanced risk for UTIs in patients with DM include age, metabolic control, duration of DM, diabetic cystopathy, more frequent hospitalization and instrumentation of the urinary tract, recurrent vaginitis and vascular complications.[10,28] However, different studies show conflicting results. Moreover, most of them do not differentiate between patients with DM type 1 and DM type 2.

Before, we have determined the risk factors for the prevalence of ASB and the incidence of symptomatic UTIs in a large cohort of 636 diabetic women. We found that women with DM type 1 with a longer duration of diabetes, or the presence of peripheral neuropathy and macroalbuminuria, had an increased risk of ASB. In women with DM type 2, a higher age, macroalbuminuria, and a recent symptomatic UTI predisposed for ASB. There was no association between the diabetes regulation

and the presence of ASB.[8] Equally to healthy women, the most important risk factor for the development of a symptomatic UTI for women with DM type 1 was recent sexual intercourse. For women with DM type 2, the most important risk factor of a symptomatic UTI was the presence of ASB.[17,29] Thirty-four percent of the women with DM type 2 and ASB developed a symptomatic UTI compared with 19% of the women without ASB.[30]
It has been suggested that diabetic cystopathy and peripheral neuropathy are associated with the pathogenesis of UTIs in diabetic patients.[10] However, others and we could not find a correlation between the presence of peripheral neuropathy and a bladder residue after micturition with the presence of ASB.[8,31,32]

3. Bacteriology

The bacteria isolated from diabetic patients with a UTI are similar to those found in nondiabetic patients with a complicated UTI.[33] As in uncomplicated UTIs, *E. coli* causes the majority of infections. However, other species are relatively more frequently cultured in these patients. For example, one study reported *E. coli* to be the causative uropathogen in 47% of the UTIs in diabetic patients and in 68% of the UTIs in nondiabetic patients.[34] Non-*E. coli* uropathogens, found in patients with DM include *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., Group B streptococci and *Enterococcus faecalis*.[7,12,28] Some authors found that diabetic patients are more likely to be infected with a resistant uropathogen.[34,35] However, we could not confirm this finding in our cohort of diabetic women with ASB (*see Chapter 5*).[36]

4. Consequences of asymptomatic bacteriuria

Recently, a large study among 796 sexually active, nonpregnant women without DM (aged 18 to 40 years) identified ASB as a strong predictor of a subsequent symptomatic UTI.[37] In other studies of nondiabetic patients, it was suggested that ASB can lead to recurrent UTIs, progressive renal impairment, hypertension and an increased mortality,[38] although most authors mean that ASB per se in a healthy individual probably causes no harm.[39,40] However, despite the high prevalence of ASB among women with DM, little is known about the consequences in this specific population.[7,12]
In the study discussed in section 2.3, we have shown that women with DM type 2 with ASB at baseline had an increased risk of developing a UTI during the 18-month follow-up compared to women with DM type 2 without ASB at baseline (17% without versus 27% with, $p = 0.02$). In contrast, we did not find a difference in the incidence of a symptomatic UTI between DM type 1 women with and without ASB. However, a more interesting finding was that women with DM type 1 and ASB had tendency to a faster decline in renal function than those without ASB (relative increase in creatinine 4.6% versus 1.5%, $p=0.2$).[30] If longer follow-up studies (*as the study in Chapter 11*) show that ASB contributes to the development of diabetic nephropathy, this would have important consequences. Diabetes now

accounts for 35% of all new cases of end-stage renal disease in the United States, and persons with DM make up the fastest growing group of renal dialysis and transplant recipients.[41,42]

5. Clinical presentation

UTIs in patients with DM can be either asymptomatic or symptomatic. ASB is defined as the presence of at least 10^5 colony forming units of the same urinary tract pathogen per milliliter in two consecutive cleanvoided midstream urine cultures. Several studies have shown that the presence of ASB is a predictor of symptomatic infections in patients with as well as in patients without DM.[17,37]

The presentation of a lower (symptomatic) UTI can be accompanied by classical symptoms as dysuria, frequency, urgency, haematuria, and/or abdominal discomfort. However, the same symptoms may be produced by inflammation in the urethra or by infective agents as *Chlamydia trachomatis*, herpes simplex or by a vaginitis (e.g. *Candida albicans* infection), which also occur frequently in women with DM. Therefore a urine specimen should be checked for leukocyturia (the presence, in uncentrifuged urine, of ≥5 leukocytes/high power field or 10 leucocytes/mm^3) and bacteriuria.

Upper tract involvement is common in patients with DM.[9,43] Acute pyelonephritis is a clinical syndrome characterized by fever and chills, flank pain, costovertebral angle tenderness and other general symptoms, such as nausea and vomiting. There may or may not be symptoms of lower UTI, such as dysuria. However, some patients only present with symptoms of a lower UTI but nevertheless have upper tract involvement (subclinical pyelonephritis).[10] Bilateral involvement is more common in diabetic patients.[44] Infection leads to bacteraemia relatively often in these patients.

There are exceptional cases of renal abscesses, papillary necrosis and emphysematous pyelonephritis.[12,45] Renal abscess formation should be suspected in patients who do not respond to antimicrobial therapy after 72 hours. Therefore, if symptoms do not resolve within this time period, ultrasonography or computed tomography (CT) scanning of the kidneys should be performed.[10] Papillary necrosis is also a complication of UTI in diabetic patient that is important to recognize. Symptoms consist of flank pain, chills, fever and renal insufficiency develops in 15% of the cases. Therefore the diagnosis should be suspected in patients responding poorly to antimicrobial therapy. Emphysematous pyelonephritis is a necrotizing infection characterized by gas production within the renal parenchyma. The disease is seen almost exclusively in diabetic patients. Gram negative bacteria are the most common isolates but multiple organisms occur. Clinical features include fever, flank pain and a palpable mass in 45% of the patients. Bacteraemia is a frequent complication of emphysematous pyelonephritis. Diagnosis is made radiographically, starting with a plain abdominal film of the kidney, ureter and bladder that detects renal emphysema in 85%. Ultrasound can be useful, especially in diagnosing obstructive complications. However, CT-scanning (without contrast)

is the study of choice because of its high sensitivity and because it precisely defines the localization and extension of the gas formation , which is important in determining the optimal therapeutic strategy.[10]

6. Treatment

Despite the high prevalence of the disease, clinical trials specifically dealing with the treatment of UTIs in diabetic patients are rare. No randomized trials are available comparing the optimal duration and the choice of the treatment. Therefore, most recommendations for treatment of UTIs in diabetic patients are based on expert opinions more than on scientific evidence.

There is debate about whether all UTIs in patients with DM should be considered and subsequently treated as complicated infections. Do the vast majority of UTIs in diabetic patients need to be labeled 'complicated' with the resulting more aggressive management? Why not be more conservative, get the data from prospective studies and not create 'disease' when there is none in many patients? Some authors indeed state that the term 'complicated' should be reserved for (diabetic) patients with therapy failure (persistent or recurrent infection) or with the presence of other conditions which in itself would lead to categorization as 'complicated UTI' (e.g. abnormalities of urinary tract, impaired renal function).[46,47] However, others[35,48] state that all UTIs in patients with DM should be treated as complicated infections, in order to avoid the development of possible dangerous complications.

6.1 Antibacterial treatment

Few clinical trials have dealt with the outcome of treatment of ASB in patients with DM.[9,43,49] From these studies, the authors conclude that a) two weeks of treatment is as effective as 6 weeks treatment; b) the recurrence rate is high, even after prolonged antibiotic treatment; and c) recurrences (four to eight weeks post-therapy) are mostly reinfections and not relapses with the same microorganism (which occur earlier). In addition, physicians should be aware of the high prevalence of underlying structural genitourinary abnormalities among bacteriuric women with DM.[43]

The need for screening of ASB in diabetic (female) patients, with the intention to treat, depends on the question whether or not ASB per se can lead to serious complications as renal function deterioration.[50] Since such evidence is not yet available, we and several authors,[39] but not all,[10,40] believe that a restrictive policy towards the treatment of ASB is justified. Especially since it is not known whether treatment of ASB in women with DM leads to an improved outcome,[33] but also because of the possible side effects of the antibacterial therapy and the increasing antimicrobial resistance rate. However, physicians must be aware of the potential of underlying pathology and serious complications.[43,51]

For uncomplicated acute bacterial cystitis (that is, in otherwise healthy young women) the Infectious Diseases Society of America (IDSA) recommends a 3 day

course with trimethoprim-sulfamethoxazole (TMP-SMX) as standard therapy. Alternatively, trimethoprim alone or a fluoroquinolone, for example ofloxacin, can be prescribed. Other fluoroquinolones have similar effectiveness, but regarding the higher costs and the increasing problem of resistant microorganisms, these should only be used as an alternative in communities with high rates of resistance to TMP-SMX.[52] However, the IDSA guidelines do not include complicated infections.

Few therapeutic trials have specifically been performed among patients with DM. Because of the frequent (asymptomatic) upper tract involvement and the possible serious complications, many experts recommend a 7- to 14-day oral antimicrobial regimen for bacterial cystitis in diabetic patients, in stead of the recommended 3-day course for uncomplicated cystitis.[10,29,53] In a recent double-blind study, the efficacy in the treatment of complicated urinary lower UTIs of a 5-day course of ofloxacin was compared to a 10-day regimen. Four hundred and sixteen women were studied of whom an unknown percentage had DM. The authors concluded that both regimens were equally effective.[54]

Although some authors state that in diabetic patients the choice of agent does not differ from the treatment in otherwise healthy patients,[33,47] most authors prefer antibacterial agents that achieve high levels not only in the urine but also in the urinary tract tissues, for example fluoroquinolones, TMP-SMX and amoxicillin-clavulanic acid.[29,55] This may especially hold true given the recent data indicating invasion of *E. coli* into the bladder cells.[26] A randomized, double-blind study including 85 (20%) women with DM has shown that a 7-day regimen with ciprofloxacin or with ofloxacin both result in a cure rate of 97% five to nine days after treatment of a complicated lower UTI.[56] In general, nephrotoxic antibacterial agents should be avoided whenever possible. As stated earlier in this section, evidence for either optimal duration of therapy or choice of antibacterial agent is lacking. Noteworthy is the possible hypoglycaemic effect of TMP-SMX, which has been observed using (larger doses of) this agent.[53,55]

In all cases of suspected pyelonephritis in diabetic patients, a culture of urine before starting therapy is indicated as well as blood cultures if the patient is severely ill.[10] The treatment of uncomplicated pyelonephritis does not differ for patients with or without DM. For treatment of mild acute pyelonephritis the IDSA recommends an oral fluoroquinolone, possibly after an initial single parenteral dose of an antibacterial. Diabetic patients are usually treated within the hospital, with a parenteral fluoroquinolone or a cefalosporine as initial therapy. In communities with a resistance rate of <15% of *E. coli* to TMP-SMX, this agent is considered a suitable alternative. If symptoms have resolved, after 48 to 72 hours, oral therapy may be started. These recommendations rely on clinical practice, since all randomized studies comparing oral with intravenous therapy have excluded patients with underlying systemic illnesses, such as DM. The current standard duration of therapy for uncomplicated pyelonephritis in both diabetic as in nondiabetic patients is fourteen days.[29,51,55,57] In a recent randomized trial a 7-day oral ciprofloxacin regimen was more effective than a 14-day TMP-SMX regimen for the treatment of uncomplicated pyelonephritis, as indicated by greater bacteriologic and clinical

cure rates.[58] This was probably due to a high resistance rate (18%) to TMP-SMX in this study. However this study does indicate that in uncomplicated pyelonephritis a treatment duration of seven days is enough. Although highly interesting, comparable studies will have to be performed specifically enrolling patients with DM, before such a regimen can be advised in these patients.

In patients with DM, a follow-up urine culture (two to four weeks post-therapy) is considered useful to detect early relapses and because of the higher treatment failure.[35] It is clear from the discussion in this section that clinical trials specifically dealing with the treatment of UTIs in diabetic patients, comparing the optimal duration and the choice of the therapy, are needed.

The traditional treatment of emphysematous pyelonephritis is nephrectomy of the affected kidney. Surgery has been reported to lower the mortality from 80% in patients treated with antimicrobial treatment alone, to 20%.[10] Although an increasing number of cases are reported of successful conservative management, antibacterial therapy combined with percutaneous drainage,[59] no consensus exists as to whether this strategy should replace (or proceed) the standard nephrectomy.

6.2 Non-antibacterial treatments and preventive strategies

The worldwide increasing problem of resistant uropathogens[60] calls for additional non-antibacterial strategies, both for the treatment and for the prevention of UTIs (*Table 2*). General advice includes sufficient fluid intake, complete emptying of the bladder during voiding, less use of spermacides, and restrictive catheter use.

An interesting possible preventive or treatment option is ingestion of cranberry juice. At first, the beneficial effect of cranberry juice was thought to be the result of acidification of the urine. More recently, *in vitro* studies have identified the inhibition of bacterial adherence to the uroepithelial cells as the most plausible

Table 2 Non-antimicrobial treatments and strategies that possibly reduce the incidence of urinary tract infections[a]

General preventive strategies:[61] sufficient fluid intake complete emptying of bladder during voiding less use of spermacides restrictive catheter use[62]
Cranberry juice (oral)[63]
Lactobacilli (oral or vaginal)[63,64]
Estrogen suppletion in postmenopausal women (oral or vaginal)[65-67]
Vaccines: Urovac[68] FimH-adhesin-based (under development)[69,70]

[a] the strategies mentioned have been studied in patients without DM

mechanism of action.[71] Another possible preventive strategy is the oral or vaginal administration of lactobacilli. Lactobacilli are part of the commensal vaginal flora and are thought to protect against UTIs by competitive exclusion of uropathogens.[72] In a randomized trial, regular drinking of cranberry juice but not of lactobacillus GG drink reduced the recurrence of UTIs in women with *E. coli* infection.[63]

In addition, several investigators have studied the influence of estrogen administration. Estrogen deficiency in postmenopausal women has been implicated in the pathogenesis of recurrent UTI, apparently as a result of an increase in vaginal pH and the subsequent reduction in the number of lactobacilli.[66] Several randomized trials of estrogen administration have been performed, most including only small numbers of patients and with conflicting results. In a recent review, the authors conclude that estrogen administration is of benefit in decreasing the recurrence rate of UTIs in postmenopausal women, especially if administered vaginally.[65] A randomized, blinded study among 2763 postmenopausal women who participated in a study on coronary heart disease, reported no reduction of the frequency of UTIs in patients with oral hormone therapy (estrogen plus medroxyprogesterone) compared to women who received a placebo.[67]

All strategies mentioned have been studied in nondiabetic patients but we think that the results will be comparable in patients with DM.

Since the adherence of *E. coli* to the uroepithelial cell is an essential step in the pathogenesis of UTIs, prevention of this would theoretically lead to a decreased incidence of UTIs. Therefore, the current development of a vaccine, based on the FimH adhesin of type 1 fimbriae of *E. coli* is very promising. *In vitro* and animal studies have shown that this vaccine can prevent adherence of *E. coli* to uroepithelial cells and decrease incidence of UTIs in vaccinated monkeys.[69,73] We have demonstrated that addition of vaccine-induced antiserum to uroepithelial cells isolated from diabetic women, also decreases the adherence of type 1-fimbriated *E. coli* to diabetic uroepithelial cells (*see Chapter 4*).[70] In addition, another vaccine is being studied in women with recurrent UTIs. This vaccine is based on immunization by vaginal suppositories containing heat-killed uropathogenic bacteria from 10 different isolates.[68] If proven effective, these vaccines would be a welcome supplement of our therapeutic armamentarium.

In the last years, more research has been done in the area of prevention of post-operative infections in diabetic patients. Although non-randomized, these studies confirm the hypothesis that hyperglycemia is associated with an increased risk of post-operative infection. The authors recommend optimal peri-operative glycaemic control (glucose levels <200 mg/dl).[47,74]

7. Future issues

Longer follow-up studies among diabetic patients (as the study described in *Chapter 11*) analyzing the effects of ASB on renal function should answer the question whether women with DM should be kept nonbacteriuric (see also the General Discussion in *Chapter 12*). Furthermore, randomized therapeutic trials

specifically enrolling patients with DM will have to define the best therapeutic management, focussing on type of antimicrobial agent and optimal treatment duration. New developments on non-antibacterial approaches must show their value in preventing UTIs in diabetic patients.

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Three

Adherence of type 1-fimbriated *Escherichia coli* to uroepithelial cells: more in diabetic women than in control subjects

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Diabetes Care 2002;25(8):1405-9

Abstract

Introduction

Women with diabetes mellitus (DM) have bacteriuria more often than women without DM. Because *Escherichia coli* adhere better to vaginal cells of nondiabetic patients with recurrent urinary tract infections (UTIs) than to those obtained from healthy control subjects, it was hypothesized that *E. coli* adhere more to the uroepithelial cells of diabetic women, either because of substances excreted in the urine (e.g., albumin, glucose, Tamm Horsfall Protein) or because of a difference in the uroepithelial cells.

Methods

A T24 bladder cell line and uroepithelial cells of 25 diabetic women and 19 control subjects were incubated with three different *E. coli* strains.

Results

The mean numbers of type 1-fimbriated *E. coli* that adhered to diabetic and control cells were 12.9 and 6.1 ($p = 0.001$), respectively, whereas those of P-fimbriated *E. coli* were 8.8 and 8.1 ($p = 0.8$), and those of nonfimbriated *E. coli* were 2.7 and 3.4 ($p = 0.4$). The addition of various substances did not influence the adherence of *E. coli* to a T24 bladder cell line.

Conclusion

Type 1-fimbriated *E. coli* adhere more to diabetic than to control uroepithelial cells.

Introduction

Diabetic women have bacteriuria more often than nondiabetic women.[1-3] The cause of this increased prevalence however, is not yet clear. One factor may be microbial adherence, because the adherence of microorganisms to host cells is an important step in the pathogenesis of many infections. The adherence of *Escherichia coli* (the most common causative microorganism in bacteriuria) to uroepithelial cells, for example, is the first step in the pathogenesis of urinary tract infections (UTIs).[4] *In vitro* studies have shown that *E. coli* adhere better to vaginal cells obtained from nondiabetic patients with recurrent UTIs than to those obtained from healthy control subjects.[5] Other investigators have shown that *Candida albicans* adheres better to buccal cells isolated from patients with DM than to those isolated from nondiabetic control subjects.[6] Therefore, we hypothesized that *E. coli* might adhere better to uroepithelial cells isolated from diabetic women than to cells from nondiabetic control subjects.

Since various substances (e.g., glucose and albumin) are present in the urine of diabetic patients, we were interested in investigating whether these substances influence the adherence of *E. coli* to uroepithelial cells. An important substance is the Tamm Horsfall Protein (THP), a large glycosylated polypeptide produced by renal tubular cells and excreted via the urine in daily amounts of 20—200 mg.[7] Studies have shown that the binding of THP to type 1 fimbriae of *E. coli* results in a decreased binding of *E. coli* to uroepithelial cells.[8,9] Elderly men and women, a group of patients prone to develop bacteriuria, have a decreased secretion of THP in their urine.[10] Diabetic patient also have a decreased THP production. Moreover, their THP might have a different chemical composition (a higher glucose content).[11-13] The latter might lead to an altered binding of THP to *E. coli* and, consequently, to decreased or increased adherence of *E. coli* to uroepithelial cells.

The aims of the present study were: 1) to investigate the adherence of *E. coli* to the uroepithelial cells isolated from women with DM and compare it to the adherence of *E. coli* to cells isolated from women without DM, and 2) to investigate the influence of THP and a few other substances on the adherence of *E. coli* to a T24 bladder cell line.

Research design and methods

Study population

Diabetic women were recruited from the outpatient clinic of our hospital, while the nondiabetic women (control subjects) were laboratory employees. None of the women had a history of recurrent UTIs, had used antimicrobial therapy in the fourteen days before recruitment, or was pregnant at the moment of urine collection. The uroepithelial cells, obtained from one or two diabetic women and one control subject, were simultaneously investigated during each experiment.

Collection of uroepithelial cells

Uroepithelial cells were obtained from the urine (collected over a 24-h period) by centrifugation (250 x g, 10 min), stored at 4°C in Isocove's Modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies, Breda, the Netherlands), supplemented with 5% fetal calf serum (FCS) (Gibco) and 0.01 mg/ml gentamycin, and used within 24 h of collection. The cells were recovered by centrifugation (250 x g, 10 min) before the experiment, and resuspended in IMDM. This centrifugation/resuspension process was repeated four times. The number of cells was calculated by direct light microscopy using a Bürker chamber.

Bacterial strains

Three *E. coli* strains (all clinical isolates) were used: MS-,MR+ (O18K-H-, genotype- and phenotype-positive for P fimbriae and G-II adhesin, genotype-positive and phenotype-negative for type 1 fimbriae), MS+,MR- (O8K-Hnontypeable, genotype- and phenotype-positive for type 1 fimbriae, genotype- and phenotype-negative for P fimbriae), and MS-,MR- (O17K-H-, genotype- and phenotype-negative for afimbrial adhesion and type 1, S, and P fimbriae). The phenotypic expression of type 1 fimbriae was measured by the occurrence of mannose-sensitive hemagglutination (MSHA), while that of P fimbriae was measured by mannose-resistant hemagglutination (MRHA).[14] Suspensions of 10% human erythrocytes and 10% guinea pig erythrocytes in PBS were used for MRHA and MSHA, respectively. Strains were cultured overnight either in Luria broth (LB) (MS+,MR- and MS-,MR-) or on blood agar plates (MS-,MR+) to maximize the expression of type 1 and P fimbriae, respectively. Bacteria were then centrifuged (2,510 x g, 10 min) and resuspended in phosphate buffered solution (PBS). The initial inoculum (2–5 x 10^8/ml) for the experiment was measured by optical density (Dr Lange photometer, Berlin, Germany).

Purification of P fimbriae

E. coli expressing P fimbriae (MS-,MR+) were grown overnight on blood agar plates at 37°C and suspended in PBS. The bacteria were collected by centrifuging for 10 min at 2,510 x g and washed twice with PBS. The bacterial pellet was then suspended in PBS/4 mol/l ureum, placed in a 60°C waterbath for 30 min, and left to cool gradually. Next, the suspension was ultracentrifuged for 20 min at 20,000 rpm at 40°C (Centrikon T-2000, Beun-de Ronde B.V., Abcoude, the Netherlands) to mechanically remove the fimbriae from the bacteria. The supernatant was then ultracentrifuged overnight at 40,000 rpm at 4°C. The pellets were suspended in PBS/0.4% SDS and incubated for 30 min at 40°C, followed by centrifugation overnight at 40,000 rpm at 16°C. The pellets were subsequently resuspended in PBS, 4% PEG6000, and 0.5 mol/l NaCl. This suspension was incubated on ice for 1 h and centrifuged for 10 min at 9,500 x g, after which the pellet was suspended in PBS and kept at –20°C until further use. The purity of the fimbriae was confirmed by SDS-PAGE analysis.

Adherence assay uroepithelial cells

For each experiment, 2 x 10^5 uroepithelial cells were incubated with either 1 ml of 10^8/ml bacterial suspension or 1 ml PBS (negative control), with shaking 350 motilities/min for 1 h at 37°C. After incubation, the suspension was washed four times (250 x g, 10 min) with PBS to remove any unattached bacteria. A portion of the final cell suspension was then dried, fixed on a microscope slide, and Diff-Quik-stained (Dade Diagnostika GmbH, Düdingen, Switzerland). Cell suspensions with adherent bacteria were examined with oil-immersion light microscopy (x 400). The number of *E. coli* adhering to each of the first 50 uroepithelial cells was counted. The investigator (ECvL) was blinded with regard to the patient group from which the cells were isolated. Epithelial cells that overlapped other cells were excluded from evaluation. The mean number of *E. coli* per cell was calculated. All experiments were performed in duplicate, and the cells of diabetic women were tested pairwise with uroepithelial cells of nondiabetic women.

T24 cell line

T24 cell line (human bladder)[15] was cultured in IMDM supplemented with 10% FCS and gentamycin in cell culture flasks (Coining Costar, Badhoevedorp, the Netherlands) at 37°C in 5% CO_2 atmosphere. Three days before the experiment, the cells were removed from the flasks using a trypsin treatment (1 ml 0.05%, 10 min) (Gibco BRL Life Technologies) and subsequently cultured on Lab-Tek Chamber slides (Nalge Nunc. International, Naperville, IL, USA) until confluent. Immediately before the adherence experiment, the medium was aspirated and replaced by either Dulbecco's Modified Eagle Medium (DMEM) without glucose or DMEM with an additional substance. The additional substances were either glucose (0.5, 1, 5, 10 and 50 mg/ml) (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands), or human albumin (0.25 and 1.5 mg/ml) (Sigma-Aldrich Chemie BV). All experiments were performed in duplicate and repeated four times. The cell line was checked regularly for infection with *Mycoplasma*.

Adherence assay T24 cell line

For each experiment, all Lab-Tek Chamber slides were incubated with 300 µl of 10^9/ml bacterial suspension (with, respectively, MS-,MR+, MS+,MR-, or MS-,MR-) in DMEM, without glucose (negative control), with glucose, with albumin, or with THP (isolated from diabetic or nondiabetic women), respectively, for 30 minutes at 37°C. After incubation, the slides were washed four times with DMEM to remove any unattached bacteria. All slides were Diff-Quik-stained. Cell suspensions with adherent bacteria were examined with oil-immersion light microscopy (x 1,000). The investigator (ECB) was blinded with regard to the added substance. The mean number of *E. coli* per 100 cells was calculated. All experiments were performed in duplicate, and the chamber slides with the different substances were tested at the same time.

Isolation of Tamm Horsfall Protein

THP was isolated according to Tamm and Horsfall[16] from the urine of diabetic and nondiabetic women. As stated above, the urine had been collected during a 24-h period. The isolated protein was tested for purity by SDS-PAGE using a 10% polyacrylamide gel and found to have a molecular weight of 92 kD, as described in the literature.[7] Using BCA protein assay (Pierce, Rockford, IL, USA) the following THP concentrations were made: 1, 3, 10, 30, and 100 μg/ml.

Secretor status

The secretor status of the women was determined using the method described by Mollison et al.[17] A blood sample was taken from eight diabetic and nine nondiabetic women who wanted to participate and whose urine was used for the isolation of uroepithelial cells. These women were classified according to their Lewis blood group type. Those with the red cell phenotype Le(a + b –) were classified as nonsecretors of ABH substance and those with the red cell phenotype Le(a – b +) as secretors. The secretor status of women with the recessive phenotype Le(a – b –) was not further determined.

Statistics

Since the distribution was normal, differences in the adherence of *E. coli* to the uroepithelial cells from diabetic women and from control subjects, as well as differences in THP isolated from diabetics and from control subjects, were tested using the unpaired t-test. The paired t-test was used to calculate the differences in adherence before and after the addition of various substances (glucose, albumin, and THP) to the T24 cell line. Linear regression analysis was used to calculate the correlations between adherence and age, duration of the DM, glycosylated hemoglobin (GHb) and creatinine. $P < 0.05$ was considered statistically significant.

Results

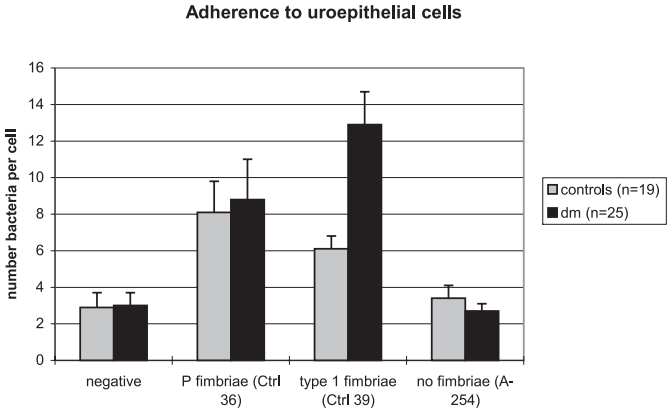
Study population

A total of 27 women with and 20 women without DM were eligible for the study. The uroepithelial cells from one control subject and two diabetic women, however, were not acceptable for the experiments, because many adherent bacteria (>10/cell) were present before the experiment had been started. Therefore, we used uroepithelial cells from 25 women with DM (age 55.5 ± 3.0 years, GHb 7.7% ± 0.3, duration of DM 17.8 ± 4.2 years, mean ± SE; of these women, 40% had DM type 1, 40% had retinopathy, 20% had neuropathy, 24% had microalbuminuria) and 19 women without DM (age 38.2 ± 3.3 years). THP was isolated from the urine of eight women with DM (age 56.5 ± 1.8 years, GHb 7.7% ± 0.1) and seven women without DM (age 42.6 ± 1.7 years).

Adherence of *E. coli* to uroepithelial cells

The number of type 1-fimbriated *E. coli* (MS+,MR-) adhering to the uroepithelial cells isolated from diabetic women was twice as high as the number in the control subjects (*Figure 1*). The mean number of *E. coli* (MS+,MR-) per diabetic cell was 12.9 ± 1.8, compared with 6.1 ± 0.8 per control cell (p = 0.001). The level of adherence ranged from 2 to 33 (median 12.5) bacteria in DM patients and from 2 to 17 (median 5) bacteria in the control subjects. This difference in adherence was not found for the strain expressing P fimbriae or the afimbrial strain (*Figure 1*).

Figure 1

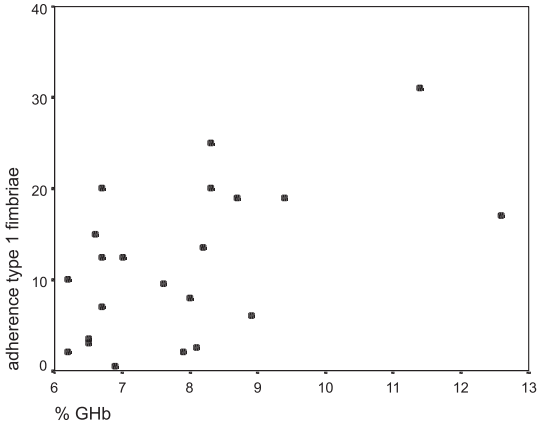


Number of bacteria per cell of three ***E. coli*** strains (MS-,MR+ with P fimbriae, MS+,MR- with type 1 fimbriae, and MS-,MR- without fimbriae) and PBS (negative control). Data are means ± SE. The uroepithelial cells were isolated from women with diabetes mellitus (DM) and without DM (controls).

We also tested whether the increased adherence of type 1-fimbriated *E. coli* could be diminished by adding isolated P fimbriae. We measured the adherence of MS+,MR- to the uroepithelial cells of two diabetic patients after the addition of different concentrations of isolated P fimbriae (1, 10, and 50 μg/ml): no decrease in adherence was found (data not shown).

The degree of bacterial adherence of type 1-fimbriated *E. coli* was correlated with GHb (p = 0.009) (*Figure 2*). In other words, well-controlled DM patients (low GHb) showed less adherence of type 1-fimbriated *E. coli* to the uroepithelial cells than poorly controlled DM patients. No correlations were found between the adherence of the *E. coli* strains and age, postmenopausal status, or the duration or presence of secondary DM complications (e.g., retinopathy, neuropathy, and microalbuminuria). Also, no differences in adherence were noted between women with DM type 1 or DM type 2.

Figure 2



The GHb (%, x-axis) was correlated with the degree of bacterial adherence of type 1-fimbriated *E. coli* (mean number per cell, y-axis) ($p = 0.009$, regression coefficient 0.5).

Adherence to T24 bladder cell line

The mean number of MS-,MR+ (geno- and phenotypically P fimbriae positive) was 267 ± 33 per 100 cells, the number of MS+,MR- (geno- and phenotypically type 1 fimbriae positive) was 168 ± 22 per 100 cells, and the number of MS-,MR- (no fimbriae) was 74 ± 16 per 100 cells before the addition of the various substances. No significant differences were found for either type 1- or P-fimbriated *E. coli* after the addition of the different concentrations of glucose or albumin (all $p > 0.3$). As a positive control, the adherence of MS+,MR- and MS-,MR+ was measured after the addition of mannose in different concentrations (0.1—50 mg/ml).[18] As expected, adherence decreased in a dose-response relationship from 82 to 15 bacteria per 100 cells for MS+,MR- (phenotype: type 1 fimbriae positive) and remained unchanged for MS-,MR+ (phenotype: P fimbriae positive). Physiological concentrations of THP (1, 3, 10, 30, and 100 μg/ml), isolated from either diabetic or control women, did not inhibit the mean adherence of MS+,MR- to the T24 cell line (all $p > 0.2$). No differences could be demonstrated between the THP isolated from diabetic women and that from nondiabetic women (all $p > 0.2$).

Secretor status

All of the diabetic women tested were secretors. Of the nine control women tested, six were secretors. The remaining three had the recessive phenotype Le(a – b –) and were not further classified as secretor or nonsecretor.

Discussion

The aim of this study was to determine the adherence of *E. coli* to uroepithelial cells isolated from women with and without DM and the influence of glucose, albumin, and THP on *E. coli* adherence to a T24 bladder cell line. We found that type 1-fimbriated *E. coli* adhered to the uroepithelial cells from diabetic women twice as well as to those from nondiabetic control subjects. We also demonstrated that different concentrations of glucose, albumin, or THP did not inhibit the adherence of either type 1- or P-fimbriated *E. coli* to a T24 bladder cell line. Furthermore, no differences in adherence could be detected after the addition of THP isolated from either diabetic or control women.

Relationships between diseases and the adherence of microorganisms to patient cells have been investigated before.[6,19,20] In general, it was found that microorganisms adhere more to (buccal) epithelial cells isolated from patients who either have a chronic disease or smoke[6,19] and to infected human epithelial (HEp-2) cell lines[20] than to cells from healthy control subjects or noninfected cell lines. The same increase has been described in studies on vaginal, buccal, uroepithelial, and periurethral cells isolated from patients (children, girls, and women) with recurrent UTIs.[5,21,22] This increased adherence of uropathogens to the cells of patients has been considered an important factor in the pathogenesis of recurrent UTIs. The increased adherence of type 1-fimbriated *E. coli* to diabetic uroepithelial cells found in the present study might also be a good explanation for the increased prevalence of asymptomatic bacteriuria (ASB) in women with DM, since these fimbriae are the most prevalent virulence factor of *E. coli* (86% genotypically and 59% phenotypically positive for type 1 fimbriae) isolated from nondiabetic and diabetic patients with ASB.[14,23]

Because the uroepithelial cells were used in solution, and no other substances (albumin, P fimbriae, glucose, and THP) inhibited the adherence of *E. coli* with type 1 fimbriae, our results suggest that the increased binding of type 1-fimbriated *E. coli* to diabetic uroepithelial cells is caused by a difference between the receptor for type 1 fimbriae on diabetic and nondiabetic uroepithelial cells. This is supported by Weinmeister and Dal Nogare[24], who showed that the receptors of severely ill (mechanically ventilated, intensive care unit) patients have a decreased amount of sialic acid and galactose on buccal cells compared with those of healthy control subjects. Those authors suggested that the altered receptor on the epithelial cells of the upper respiratory tract might explain the high prevalence of Gram negative bacterial colonization and pneumonia in the critically ill patients. At this moment, however, we do not know what the difference is between the receptors for type 1 fimbriae: are the receptors on diabetic uroepithelial cells present in a higher density, or do they have a different composition than those on nondiabetic uroepithelial cells? In the present study, we did find a correlation between GHb and the adherence of type 1 fimbriae. Because receptors for type 1 fimbriae of *E. coli* are glycoproteins (uroplakins that line the bladder mucosa)[25], we can hypothesize that diabetic uroepithelial cells have a different glycosylation of the receptor on their

cells, which results in a higher adherence capacity.

In concordance with other adherence studies[5,21,26], we found no relationship between the ability of a female subject's cells to bind bacteria and her age. It has been suggested that bacterial adherence may also be affected by the blood group antigens, which are found on the surface of uroepithelial cells. Individuals with the Lewis blood group phenotype Le(a–b+) secrete Le^b and A, B or H substances in their saliva and plasma and are called "secretors", whereas "nonsecretors" with the Le(a+b–) phenotype do have Le^a antigens in their secretions, but not A, B or H substances. Several studies have shown a correlation between the Lewis blood group phenotypes and recurrent UTIs in adult women[27,28]: nonsecretors have a higher risk of recurrent UTIs. When comparing diabetic and nondiabetic individuals, other authors found similar numbers of individuals who secreted blood group substances.[6] We determined the secretor status of a randomly chosen sample of diabetic and control women whose uroepithelial cells had been isolated for the adherence part of the study. Because all diabetic women tested were secretors, the increased adherence we found is not likely be caused by an increased frequency of the Lewis blood group nonsecretor phenotype.

The present study showed that THP had no inhibitory effect when used in physiological concentrations. Other authors have reported inhibitory effect when high concentrations (> 250 μg/ml) are used.[8,9] Such concentrations are not physiological, however, because normal THP excretion is between 20 and 200 mg/24 h. We did not find any differences between the THP isolated from diabetic women and that from control women. Therefore, it is not probable that a difference between diabetic and nondiabetic THP plays a role in the pathogenesis of the increased prevalence of bacteriuria in women with DM.

In conclusion *E. coli* with type 1 fimbriae adhere to the uroepithelial cells from diabetic women twice as well as to those from nondiabetic women. This adherence is related to the regulation of diabetes. This mechanism may play a role in the pathogenesis and increased prevalence of bacteriuria in diabetic women.

Acknowledgments

This study was supported by a grant from the Dutch Diabetes Fund (number 95.123)

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Four

FimCH antiserum inhibits adherence of *Escherichia coli* to cells collected by voided urine specimens of diabetic women

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J Urol 2004;171(4):1589-93

Abstract

Introduction

With the increasing problem of resistance in pathogenic microorganisms the development of nonantimicrobial therapies is important. Diabetes mellitus (DM) is associated with an increased incidence of urinary tract infections (UTIs). The majority of *Escherichia coli* strains, which is the most prevalent uropathogen, have type 1 fimbriae that bind to uroplakin in the bladder, as mediated by the adhesin FimH. A vaccine is being developed based on FimH.

Methods

The sequence of the FimH adhesin of 29 *E. coli* strains isolated from women with DM was determined. For adherence experiments we used *E. coli* isolated from women with DM and a T24 bladder cell line as well as the two well-defined type 1-fimbriated *E. coli* strains Ctrl 39 and NU14, and uroepithelial cells from women with DM.

Results

The *fimH* sequence of *E. coli* strains isolated from women with DM was highly homologous to the known *fimH* sequence of *E. coli* from patients without DM. Adherence assays in a T24 bladder cell line show that the adherence of these *E. coli* strains from women with DM can be inhibited by pre-incubation with antiserum raised against the chaperone-adhesin-complex FimC-FimH. Anti-FimCH antiserum also inhibited the adherence of two well-defined *E. coli* strains expressing type 1 fimbriae, NU14 and Ctrl 39, but not of the FimH mutant strain NU14H–, to uroepithelial cells from women with DM.

Conclusion

These findings suggest that a vaccine based on the FimH adhesin of type 1-fimbriated *E. coli* is a potential method of preventing UTI in women with DM.

Introduction

Women with diabetes mellitus (DM) have an increased prevalence of asymptomatic bacteriuria (ASB) and an increased incidence of symptomatic urinary tract infections (UTIs) compared to women without DM.[1,2] In our recent multicenter study, the prevalence of ASB among 636 women with DM was 26% in comparison to 6% in women without DM.[1] Women with DM type 2 with ASB were at increased risk for symptomatic UTI.[3] Moreover, upper tract infection is more common in patients with DM, and severe complications occur more frequently.[2] Several hypotheses about the etiology of the increased prevalence of UTIs in diabetic patients exist, including diabetic cystopathy, and glycosuria or abnormal leukocyte functions.[4,5] As a possible explanation, we recently described that the adherence (the first step in the pathogenesis of UTI) of *Escherichia coli*, expressing type 1 fimbriae, is higher to uroepithelial cells of women with DM compared to the adherence to uroepithelial cells of women without DM (*see Chapter 3*).[6] *E. coli* is the most common uropathogen in both patients with and without DM, and approximately 90% of uropathogenic *E. coli* strains express type 1 pili. Type 1 fimbriae are filamentous organelles that can be found at the surface of the bacterial membrane. The adhesive part of type 1 pili is the FimH adhesin, located at the distal end of the pilus. FimH adhesin is highly conserved in different strains and it has an essential role in the pathogenesis of lower UTIs. Upon inoculation of *E. coli* into the bladder type 1 fimbriae bind to mannosylated glycoproteins (uroplakins) lining the bladder mucosa.[7] Hereby the bacteria are prevented from being washed out with the urine.

We reasoned that antibodies against FimH could prevent bacterial colonization and, therefore, could prevent UTIs in women with DM. A vaccine candidate against UTIs based upon the FimC-FimH complex, has recently been developed.[8] Since women with DM are prone to UTIs, possibly due to a higher adherence of type 1-fimbriated *E. coli* to their uroepithelial cells, this vaccine might be of value for the prevention of UTIs and the complications in these patients.

In this study, three hypotheses were tested: 1) We investigated whether clinical *E. coli* strains isolated from women with DM and bacteriuria posses the same genomic sequence encoding for FimH as that in strains from otherwise healthy women with single or multiple UTIs; 2) We studied whether antiserum against FimH inhibits the adherence of *E. coli* strains isolated from women with DM to a uroepithelial cell line; and 3) Most importantly we studied whether anti-FimH antiserum inhibits the binding of well established *E. coli* strains to uroepithelial cells collected from the urine of women with DM.

Methods

Polymerase Chain Reaction (PCR) analysis and sequencing

A total of 29 *E. coli* strains were collected from the urine samples of women with DM and ASB.[9] The *fimH* genes were isolated and multiplied by PCR, and deduced amino acid sequences were compared with FimH sequences of J96, a well characterized, uropathogenic *E. coli* strain.[10]

Bacterial strains

Fourteen different *E. coli* strains (a randomly chosen subset of the 29 mentioned), that is clinical strains isolated from the urine samples of diabetic women with ASB ($\geq 10^5$ colony forming units [cfu] per ml), were used in binding assays with the uroepithelial cell line. All strains were genotypically positive for type 1 fimbriae on PCR but they differed in the presence and expression of various other virulence factors, as previously described.[5] For binding assays with uroepithelial cells from women with DM, three *E. coli* strains were used, namely Ctrl 39 (a clinical strain isolated from a women with pyelonephritis), NU14 (a well established cystitis strain) and as a negative control the FimH minus mutant NU14H– (developed as previously described).[11] NU14 and Ctrl 39 are well-known strains that are type 1-fimbriated and have been shown to adhere to uroepithelial cells in a clinically significant manner.[6,12] All strains were cultured in Luria Broth and incubated statically at 37°C for 2 x 48 h with aeration to maximize type 1 fimbriae expression. Bacteria were collected by centrifugation and labeled with fluorescein isothiocyanate (FITC) as previously described.[13] The expression of type 1 fimbriae was confirmed by assessing the mannose sensitive hemagglutination (MSHA) of guinea pig erythrocytes. The bacterial concentration was measured by optical density at 660 nm (Dr Lange photometer, Berlin, Germany). Optical densities corresponded to different bacterial concentrations, which were determined by plating serial dilutions of bacteria on blood agar plates. The bacteria were stored at –70°C until further use.

Antisera

Polyclonal antibodies against respectively purified FimC, FimH and FimCH were raised in rabbits, as described previously.[11] Before use antiserum was heat inactivated and brought to different concentrations in phosphate buffered saline (PBS) supplemented with 5% fetal calf serum (FCS). It is known from previous studies that anti-FimC antiserum does not inhibit binding.[11]

T24 bladder carcinoma cell line

The human bladder cell line T24[14] was cultured in Isocoves Modified Dulbeccos Medium (IMDM) (Gibco, Life technologies, Breda, the Netherlands) supplemented with 10% FCS and 0.001% gentamicin in cell culture flasks (Coining Costar, Badhoevedorp, the Netherlands) at 37°C in a 5% CO_2 atmosphere. Cells were removed from the flasks by trypsin treatment (2 ml 0.05% for 10 min), resuspended in IMDM

with 10% FCS and brought to a concentration of 4 x 10^5 cells per ml, as calculated by direct light microscopy using a Bürcker-Türk chamber. Cells (4 x 10^4 per well) were inoculated in sterile 96-well tissue culture plate (Costar, Badhoevedorp, the Netherlands) and left to grow at 37°C in a 5% CO_2 atmosphere until confluent (two to three days). Immediately before the experiment cells were washed three times with PBS buffer containing 4% bovine serum albumin (BSA) and 1% glycerol.

Uroepithelial cells of women with or without DM

Women with DM type 1 or DM type 2 were asked to collect urine during a 12 to 24 h time period. Women who had significant bacteriuria at urine collection were excluded. Uroepithelial cells were obtained by centrifugation (250 x g, 4°C, 10 min), washed three times, and stored at 4°C (maximal 24 h) in IMDM with 5% FCS and 0.001% gentamicin until further use. Immediately before the experiment, the uroepithelial cell suspension was centrifuged at 250 x g at 4°C for 10 minutes. The pellet was resuspended in PBS/FCS 5% to a concentration of 4 x 10^5 cells per ml, as calculated by direct light microscopy using a Bürcker-Türk chamber. In our experiments, the percentage of cells of epithelial origin was more than 90%. To control our methods uroepithelial cells of two nondiabetic women were also isolated and tested.

Bacterial adherence assays

For the assays in the T24 cell line, 2.5 x 10^7 FITC-labeled *E. coli* (one of the fourteen strains collected from women with DM and ASB) were added to 50μl antiserum against FimC, FimH or FimCH at different dilutions. Since the binding of type 1 fimbriae to uroplakins is mannose-sensitive, mannose served as a positive control for inhibition. Pre-incubation occurred for 30 minutes at 37°C. The suspension was then added to a 96-well plate containing T24 cells. After a second incubation period for 60 minutes at room temperature the cells were washed three times with PBS/FCS 5% to remove the nonadherent bacteria. Fluorescence, expressed in arbitrary fluorescence units (AFU), was determined using a Cytofluor II cytofluorometer (Per Septive Biosystems Inc., Framingham, MA, USA). For assays in uroepithelial cells of diabetic and nondiabetic patients, *E. coli* (Ctlr 39, NU14 and NU14H–) was added to the different antisera against FimC, FimH or FimCH for a pre-incubation period of 30 minutes at 37°C. After the addition of uroepithelial cells (bacteria-to-cell ratio 250:1) a second incubation period of 30 minutes at 37°C followed. The cells were washed three times with PBS/FCS 5% to remove nonadherent bacteria. Mean fluorescence per cell was calculated by flow cytometry using a FACscan (Becton Dickinson, Erembodegum-Aalst, Belgium).
Since anti-FimC (the antibody against the periplasmic FimC) does not bind to *E. coli*[11] and has the same bacteria aggregation capability as anti-Fim(C)H, maximal adherence per experiment was set by the fluorescence of T24 and uroepithelial cells that were incubated with bacteria and anti-FimC antiserum, respectively, and considered 100%.

Statistics

SPSS, release 10 for Windows 98 (SPSS, Chicago, IL, USA), was used as statistical software. The one-sample t test was used to calculate differences in adherence after the addition of anti-FimC antiserum (100% adherence), and anti-FimH antiserum, anti-FimCH antiserum and mannose. The difference between the different concentrations of anti-FimH or anti-FimCH antiserum was calculated by the Mann-Whitney U test. A value of $p < 0.05$ was considered statistically significant.

Results

Genomic *fimH* sequence of *E. coli* strains of women with DM

A total of 29 *E. coli* strains from women with DM were analyzed to determine their *fimH* sequence (*Table 1*). Results were compared to the *fimH* sequence of J96, a well characterized cystitis strain. Overall the different isolates showed great homogeneity. No variations were seen in the amino acid regions known to build up the mannose binding pocket. Most of the mutations that were found have been described previously to occur in *E. coli* strains from patients without DM with single or multiple UTIs.[10]

Adherence of *E. coli* strains of women with DM after the addition of antiserum

Fourteen different *E. coli* strains isolated from the urine samples of diabetic women with ASB were genotypically positive for type 1 pili and expressed type 1 pili. Pre-incubation of bacterial strains with anti-FimH, anti-FimCH or mannose resulted in a significant decrease in adherence of all strains to T24 bladder cells *in vitro* (*Figure 1*). The mean decrease in adherence ± standard error of the mean (SE) after pre-incubation with anti-FimH antiserum diluted to a concentration of 1:200 and 1:50 was 60% ± 11% and 72% ± 9%, respectively. The mean decrease in adherence after pre-incubation with anti-FimCH antiserum diluted to a concentration of 1:200 and 1:50 was 74% ± 13% and 84% ± 4%, respectively (all $p < 0.001$).
The difference between anti-FimH and anti-FimCH antiserum was not statistically significant ($p > 0.05$).

Adherence of *E. coli* to uroepithelial cells of women with DM after the addition of antiserum

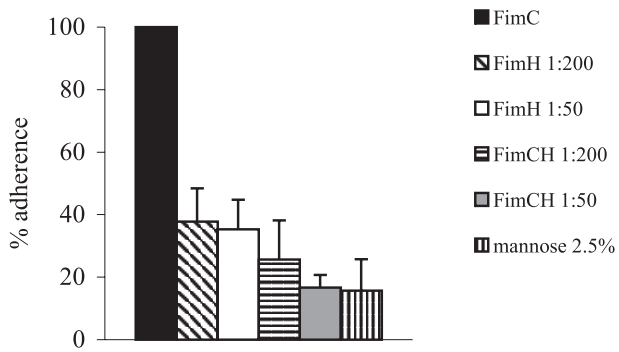
Subsequently we investigated whether the antisera against FimH and FimCH could prevent adherence to uroepithelial cells of diabetic women of the two well established type 1-fimbriated *E. coli* strains NU14 and Ctrl 39. We used the FimH minus mutant NU14H– as a negative control. Uroepithelial cells were obtained from six women with DM including three each with DM type 1 and DM type 2, with a mean age of 57 ± 19 years, mean duration of DM of 19 ± 20 years, mean haemoglobin A1c 7.3% ± 0.7%, and mean serum creatinine 90 ± 30 µmol/l. *Figure 2* shows the results. Compared to the adherence of bacteria pre-incubated with anti-FimC antiserum anti-FimH and anti-FimCH antiserum inhibited the

Table 1 Amino acid sequences of the *fimH*-allele of 29 *Escherichia coli* strains isolated from diabetic women with asymptomatic bacteriuria

					1	**1**	2	**4**		**5**	6	7	7	9	0	1	**1**		**1**	1	1	2	2	2	2
					1	**3**	7	**4**		**4**	6	0	8	2	6	9	**2**		**2**	3	6	2	4	0	4
J96	**F**	T	A	N	A	**I**	V	**C**	-	**D**	G	N	S	R	A	A	**R**	-	**F**	V	R	A	S	T	A
A294, A329, A190, A71, B9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A30,A211,A423, B1, B80, B6, C7	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C6	.	.	.	.	.	.	.	.	.	.	.	S	N	.	.	.	.	.	.	.	.	.	.	.	.	.
C27	.	.	.	.	.	.	A	.	.	.	.	S	N	.	V	.	.	.	.	.	.	.	.	.	.	.
C25	.	.	.	.	.	.	.	.	.	.	.	S	N	G	.	.	.	.	.	.	.	.	.	.	.	.
A4, A87, B4	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
A130,A67, A242	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	.	.
A234	.	.	.	.	.	.	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.
B67,B74	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.
B20	.	.	.	.	.	.	A	.	.	.	S	S	N	.	.	.	.	.	.	.	.	.	.	.	.	.
B34, B82	.	.	.	.	.	.	A	.	.	.	.	S	N	.	.	.	.	.	.	.	.	.	.	.	.	.
A33	.	P	P	D	T	.	A	.	.	.	.	S	N	.	.	.	.	.	.	A	.	.	L	.	.	.
A199	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	T

The residues listed on top are for the amino acids sequence of the FimH crystal structure of J96. The positions are numbered vertically (for example, the amino acid of strain A30 at position 27 is A = Alanine compared to V = Valine in J96). Only the deviations from the J96 sequence are shown. The gray boxes show the residues building up the mannose binding pocket.[10]

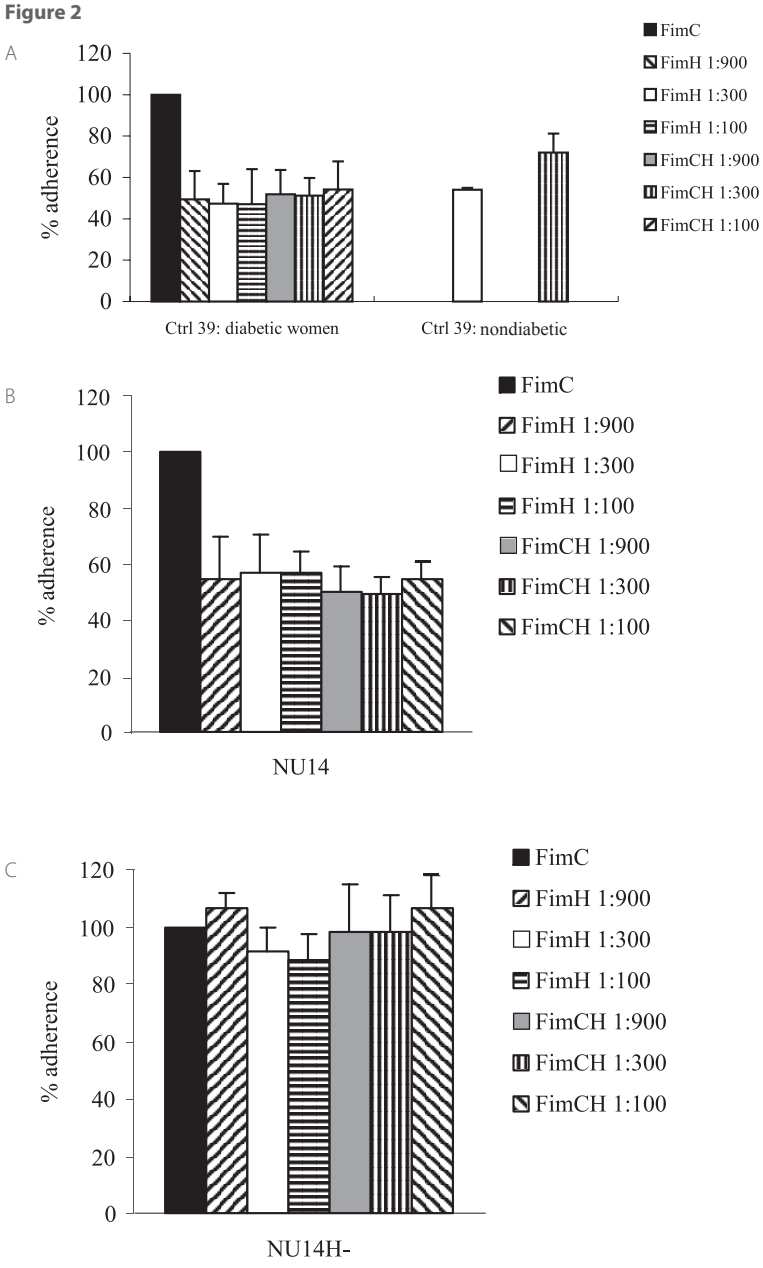
Figure 1



Mean adherence (and standard error of the mean) of 14 type 1-fimbriated *Escherichia coli* strains isolated from urine of women with diabetes mellitus and asymptomatic bacteriuria to T24 cell line after pre-incubation with anti-FimC (100%), anti-FimH or anti-FimCH antiserum, or mannose

adherence of Ctrl 39 and NU14 considerably. After pre-incubation with anti-FimH antiserum 1:900, 1:300 or 1:100 the mean decrease in adherence of Ctrl 39 was 51% ± 14% ($p = 0.07$), 53% ± 10% ($p = 0.03$) and 53% ± 17% ($p = 0.08$), respectively. After pre-incubation with anti-FimCH antiserum 1:900, 1:300 or 1:100 it was 48% ± 26% ($p = 0.02$), 49% ± 9% ($p = 0.01$) and 46% ± 14% ($p = 0.03$). For NU14 the mean decrease in adherence after pre-incubation with anti-FimH antiserum 1:900, 1:300 or 1:100 was 45% ± 15% ($p = 0.06$), 43% ± 13% ($p = 0.049$), and 43% ± 8% ($p = 0.01$). After pre-incubation with anti-FimCH antiserum 1:900, 1:300 or 1:100 it was 51% ± 9% ($p = 0.003$), 51% ± 6% ($p < 0.001$) and 46% ± 6% ($p = 0.001$). The difference between anti-FimH and anti-FimCH antiserum in the ability to prevent binding was not significant ($p > 0.05$). The FimH negative mutant NU14H– was not significantly inhibited by anti-FimH or anti-FimCH antiserum (each at different concentrations $p > 0.05$). The maximal adherence of 100%, as determined by the fluorescence of uroepithelial cells incubated with bacteria and anti-FimC antiserum, was set separately for each experiment. But, as expected, the absolute value of the fluorescence of the FimH negative mutant was lower than that of NU14. The tests were repeated with uroepithelial cells of two control subjects without DM with a mean age of 37 ± 13 years. The extent of the inhibition of adherence to the uroepithelial cells of control subjects without DM was comparable to that of uroepithelial cells of women with DM (*Figure 2a*).

Figure 2



Mean adherence (and standard error of the mean) of three *Escherichia coli* strains to uroepithelial cells of women with diabetes mellitus (DM) and 2 controls without DM after pre-incubation with anti-FimC (100%), anti-FimH or anti-FimCH antiserum.

A type 1-fimbriated Ctrl 39 in 5 preparations

B type 1-fimbriated NU14 in 6 preparations

C NU14H– (FimH minus mutant of NU14) in 6 preparations

Discussion

Women with DM have ASB more frequently than women without DM.[1] We have reported that in patients with DM ASB is associated with symptomatic UTIs,[3] and possibly also with a faster decrease in renal function.[15] Furthermore, symptomatic UTIs tend to have a more complicated course in these patients.[2] Therefore, the development of nonantimicrobial strategies could be valuable in the treatment and prevention of UTIs and their possible complications in diabetic patients. Langermann et al. studied the prevention of *E. coli* infection by systemic vaccination with the FimH adhesin.[11] Antibodies against FimH and the FimC-FimH complex prevented colonization of the bladder mucosa of mice *in vivo*. In addition, monkeys were vaccinated and subsequently challenged with *E. coli* NU14.[16] Three of four vaccinated monkeys were protected from bacteriuria compared to no control monkeys.

We have previously reported that the adherence of type 1-fimbriated *E. coli* to the uroepithelial cells of diabetic women is increased compared to that to uroepithelial cells of nondiabetic controls.[6] We hypothesized that this could be due to a difference in the uroepithelial cell receptor for type 1-fimbriated *E. coli* which are described as major glycoproteins (uroplakins),[7] and not to a difference in the causative pathogens of UTIs in diabetic women. In this study we also noted that the *fimH* sequence, especially the regions of the mannose binding pocket, of the *E. coli* isolated from the urine of diabetic women is identical to the *fimH* sequence described in the literature. As expected, we could inhibit the adherence of fourteen *E. coli* isolated from the urine of diabetic women to a T24 bladder cell line by antisera against FimH or the FimC-FimH complex. Therefore, we concluded that there are no significant differences in the FimH-mannose interactions among uropathogenic *E. coli* isolated from the urine of women with and without DM.

Since there might be a change in the uroepithelial cell receptor of diabetic women, we further investigated whether the anti-FimH and anti-FimCH antiserum could prevent *in vitro* the adherence of type 1-fimbriated *E. coli* to uroepithelial cells isolated from women with DM. Although the bacterial adherence via the uroplakins is the most likely mechanism, we did not check for it. Pre-incubation with anti-FimH or anti-FimCH resulted in a significant decrease in adherence of *E. coli* to uroepithelial cells as compared to the number of *E. coli* adhering after pre-incubation with negative control anti-FimC antiserum, which targets the cytoplasmic FimC chaperone that is not expressed on the cell surface.[17] The percent of inhibition was comparable to that of uroepithelial cells from women without DM.

An obvious difference between our study and earlier studies with this vaccine is that the degree of inhibition in adherence after the addition of antiserum was much less than the 90% to 100% as reported by Langermann et al.[16] The most likely explanation for this difference is the different target cells that were used in these studies. Langermann et al. used a human bladder J82 cell line, while we used uroepithelial cells collected from the voided urine of patients. Because these cells were not purified, the suspension consists of a various living and dead cells, and some possibly disturbing urinary components; for instance Tamm Horsfall protein

can also bind type 1 fimbriae.[6] Supporting this hypothesis, we found almost complete inhibition in our adherence experiment in the T24 bladder cell line. Other adherence studies with uroepithelial cells showed a wide range of reduction. Hagberg et al. studied the adherence of hundred type 1-fimbriated *E. coli* strains to freshly voided uroepithelial cells from a healthy volunteer after pre-incubation with mannose and found a decrease from an average of 22 to 5 bacteria per cell (77% decrease).[18] In our adherence assay in an earlier study we observed 50% decrease in adherence after the addition of mannose only,[6] comparable to the decrease that we noted for anti-FimH or anti-FimCH antiserum. Studies of the inhibition of *E. coli* adherence by cranberry juice showed inhibition that varied from less than 24% to 97%.[19] The amount of the decrease depended on the juice dilution and the *E. coli* strain. We observed no difference in ability to prevent *E. coli* binding to uroepithelial cells isolated from the urine of women with DM and control subjects without DM by anti-FimH or anti-FimCH antiserum. Therefore, even when diabetic women express modified uroepithelial cell receptors, the antisera tested can decrease the type 1 fimbriae based adherence of *E. coli* in diabetic patients.

How can we translate our results to the *in vivo* situation in diabetic women? It is difficult to make a direct comparison between the *in vitro* inhibitory activity of rabbit serum used in our assays and the *in vivo* inhibitory activity of urinary antibodies of humans. There is a difference between the IgG titers of humans compared to those of rabbits. Normally the total IgG titer of rabbits (2 to 3 mg/ml) is approximately 25% of the human serum titer (6 to 10 mg/ml). Rabbit antiserum used in our experiments is known to contain exceptionally high levels of FimH specific IgG, that is more than 20-fold higher than titers typically achieved in humans (data not shown). Furthermore, we expect that the overall serum antibody titers may be less important in protecting against UTIs than FimH specific antibody titers in urine and vaginal secretions.[16] In humans total urinary IgG is 1,000-fold lower than total serum IgG. We can speculate that it may be true for FimH specific IgG to our knowledge but exact data are lacking. For our assays we used rabbit antisera in dilutions up to 1:900. If comparable serum inhibitory activity was obtained in humans, urinary concentration that were 1,000-fold lower could indeed have clinical relevance. To date we have no direct measurements that permit us to extrapolate our results to the *in vivo* situation. Clinical studies will be required to demonstrate that the vaccine induced antibodies are inhibitory in patients with DM *in vivo*.

In summary, we found that the mannose binding pocket of the *fimH* sequence of *E. coli* isolated from urine samples of women with DM is identical to the *fimH* sequence of *E. coli* of women without DM with UTI. The adherence of these *E. coli* isolates to a T24 bladder cell line can be prevented for the most part by antiserum raised against FimH and the FimC-FimH complex. Anti-FimH and anti-FimCH antiserum significantly decreased the adherence of type 1-fimbriated *E. coli* and not of the FimH minus mutant to cells collected by voided urine specimens of diabetic women *in vitro*. A vaccine based on FimH adhesin is a potential method of preventing UTIs in women with DM.

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Five

Diabetes mellitus in itself is not a risk factor for antibiotic resistance in *Escherichia coli* isolated from patients with bacteriuria

Meiland R, Geerlings SE, De Neeling AJ, Hoepelman IM
Diabet Med 2004;21(9):1032-4

Abstract

Introduction

To investigate whether diabetes mellitus is a risk factor for resistance in *Escherichia coli* isolated from patients with bacteriuria.

Methods

Data were obtained from a multicenter study. A clean-voided midstream urine culture was collected from 636 women with diabetes mellitus (DM), who were between 18 and 75 years old, attended an outpatient department and had no symptoms of a urinary tract infection. The resistance of *E. coli* was determined for different antimicrobials. The results were compared with resistance data from routine isolates of *E. coli*, obtained from women in the same age category, time period and location.

Results

A total of 135 *E. coli* were isolated from women with DM (mean age 57 ± 14 years) and compared to 5907 routine isolates of *E. coli* obtained from female patients visiting an outpatient department (mean age 52 ± 17). The resistance rates of *E. coli* isolated from diabetic patients and the routine isolates of *E. coli* to trimethoprim-sulfamethoxazole were 19% and 23%, respectively, to amoxicillin 16% and 32%, to nitrofurantoin 1% and 3%, to ciprofloxacin 0% and 4%, to ofloxacin 0% and 5%, and to norfloxacin 1% and 4%.

Conclusion

The resistance of uropathogenic *E. coli* in non-hospitalized women with DM is not higher than that seen in routine isolates of *E. coli*. This suggests that DM in itself is not a risk factor for resistance.

Introduction

Antimicrobial resistance among uropathogens causing community-acquired urinary tract infections (UTIs) is increasing. However, little information is available on the factors associated with the risk of having a UTI with a resistant uropathogen. It has been suggested that diabetes mellitus (DM) in itself is a possible cause of resistance.[1] We previously reported a four-fold increase in prevalence of asymptomatic bacteriuria in women with diabetes.[2] Here, we report on the antibiotic resistance of the uropathogenic *Escherichia coli* isolated from these diabetic women and compare our results with the resistance rates of routine urinary isolates collected in the same period.

Methods

Between October 1996 and February 1998 a total of 636 diabetic women participated in a multicenter study in four Dutch hospitals, as described before.[2] Women were eligible for this study if they were between 18 and 75 years old, had DM type 1 or type 2 , attended an outpatient department, and had no symptoms of a UTI. They were asked to collect a clean-voided midstream urine sample. The resistance patterns of all *E. coli* strains isolated from these cultures were compared with resistance data from routine urinary isolates of *E. coli*. The latter *E. coli* strains were isolated by eight local laboratories, from urine samples of women in the same age category, visiting an outpatient department in the same time period. Repeat cultures from the same individual were excluded.

Culture was performed according to the standard procedures of the different laboratories. A positive culture was defined as the presence of at least 10^5 colony-forming units of a uropathogen per milliliter. The identification of the bacteria and their susceptibility to antimicrobial agents were determined using the Vitek automated identification system (bioMérieux, Den Bosch, the Netherlands). The resistance of *E. coli* was determined for different antimicrobials, using breakpoints from the National Committee for Clinical Laboratory Standards (NCCLS) 1998 for interpretation of the results.[3] The Minimal Inhibitory Concentration (MIC) breakpoints used for resistance (in μg/ml) are: amoxicillin ≥ 32, nitrofurantoin ≥ 128, trimethoprim-sulfamethoxazole (TMP-SMX) ≥ 4/76, ciprofloxacin ≥ 4, ofloxacin ≥ 8, and nofloxacin ≥ 16. Bacteria with intermediate resistance were considered sensitive in the analyses. Differences in resistance rates between isolates from diabetic women versus routine isolates were calculated by the Chi square test.

The study was approved by the Medical Ethics Committee of all participating hospitals, and followed the human experimentation guidelines of the University Medical Center Utrecht (Utrecht, the Netherlands). All women with DM gave written informed consent.

Results

A total of 135 *E. coli* were isolated from women with DM (mean age 57 ± 14 years) and compared with 5907 routine isolates of *E. coli* obtained from female patients visiting an outpatient department (mean age 52 ± 17). The results of the resistance patterns are summarized in *Table 1*. For all antimicrobial agents tested, the resistance rates were lower in *E. coli* isolated from urine of diabetic women than those isolated from urine samples of the general female population.

Table1 Antibiotic resistance in *Escherichia coli* isolated from urine of non-hospitalized women with diabetes mellitus versus resistance rates of routine urinary *E. coli* isolates in the Netherlands

	diabetes		routine isolates		p value
	resistance	n	resistance	n	
TMP-SMX[1]	26 (19%)	135	1328 (23%)	5748	> .05
amoxicillin	22 (16%)	135	1735 (32%)	5506	< .001
nitrofurantoin	2 (1%)	135	158 (3%)	5907	> .05
ciprofloxacin	0	134	117 (4%)	2783	< .05
ofloxacin	0	133	44 (5%)	856	< .01
norfloxacin	1 (1%)	82	172 (4%)	4381	> .05

[1] TMP-SMX indicates trimethoprim-sulfamethoxazole

Discussion

We have compared the resistance rates of *E. coli* isolated from women with DM to those of routine isolates and found no correlation between diabetes and an increased resistance in uropathogenic *E. coli*.

Some authors,[4] but not all,[5,6] have demonstrated an association between the presence of a TMP-SMX- or quinolone-resistant uropathogen and DM. Bonadio et al. compared 36 community-acquired *E. coli* strains from diabetic patients with 179 *E. coli* strains from nondiabetic patients, and found that *E. coli* from diabetic patients were more resistant to TMP-SMX and to norfloxacine, but not to ampicilline. The differences, however, were not statistically significant.[7] When we consider DM as an immunocompromising disease, we would expect less virulent uropathogens,[8] and possibly also causative microorganisms with a lower resistance rate compared with microorganisms isolated from non-immunocompromised patients. On the other hand, a higher resistance rate could be expected in uropathogens of diabetic patients because of the increased incidence of UTIs in these patients, and the subsequent increased use of antibiotics. This hypothesis is supported by the results of Brown et al, who showed that women who had recently taken TMP-SMX had a significant higher risk of infection with a resistant microorganism.[9] In addition, other factors such as frequent hospitalization, urologic instrumentation, and antimicrobial treatment for other indications than a UTI, could contribute to an

association of resistant uropathogens and DM.[4] Therefore we investigated whether the presence of DM was associated with resistant uropathogens.
The resistance rate we found towards TMP-SMX, the drug of choice for the treatment of community-acquired UTIs, is comparable to that found in the USA.[10] This high percentage (approximately 20%) precludes the usage for uncomplicated UTI and pyelonephritis without sensitivity testing.[10,11]
One limitation of our study is the comparability of the patient groups. The diabetic women participating in this study had no symptoms of a UTI. Complete data on symptoms, recent hospitalization or antimicrobial use in the control group are lacking, but one can assume that the most prevalent indication for a urine culture is a symptomatic UTI. This might have biased our results. However, we tried to avoid this by comparing our cultures with those obtained in a similar setting (the outpatient department) and by excluding repeat cultures from the same individual. Besides that, we have previously shown in this cohort of diabetic patients that asymptomatic bacteriuria precedes a symptomatic UTI[12], a phenomenon that has also been described in nondiabetic patients.[13] The routine isolates were derived from the general population visiting an outpatient department. Although diabetic patients were not specifically excluded from these, the large number will have minimized the potential effect.
In conclusion, we found that the resistance of uropathogenic *E. coli* in non-hospitalized diabetic women is not higher than in routine isolates of *E. coli*. This suggests that DM in itself is not a risk factor for resistance.

Acknowledgements

This study was supported by a grant from the Dutch Diabetes Fund (grant 95.123).

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Six

History taking and leukocyturia predict the presence of asymptomatic bacteriuria in women with diabetes mellitus

Meiland R, Geerlings SE, Stolk RP, Hoes AW, Hoepelman IM
Eur J Epidemiol 2004;19:1021-7

Abstract

Introduction

To investigate the accuracy of history taking to diagnose asymptomatic bacteriuria (ASB) in diabetic women, and the added value of leukocyturia.

Methods

Data were obtained from a multicenter study including 465 women with diabetes. Many patient characteristics were considered as potential diagnostic determinants. A urinary leukocyte count and a urine culture (the criterion standard) were performed. Logistic regression analyses were performed and areas under the receiver operating characteristic curves (AUC) were calculated.

Results

For women with DM type 1 (n = 236, ASB 11%), duration of diabetes and glycosylated hemoglobin (GHb) were powerful predictors of ASB. The AUC of the model including these 2 variables was 0.66 (95% confidence interval (CI) 0.53–0.78). After addition of leukocyturia, the AUC increased considerably to 0.78 (95% CI 0.68–0.88; p = 0.018). For women with DM type 2 (n = 229, ASB 19%), age and the number of symptomatic urinary tract infections (UTIs) in the previous year were the strongest predictors of ASB. The AUC of the model including these variables was 0.70 (95% CI 0.61–0.80). After addition of leukocyturia, the AUC increased to 0.79 (95% CI 0.71–0.86; p = 0.023).

Conclusion

In diabetic women, ASB can be diagnosed using two easily obtainable variables (duration of diabetes and GHb for women with DM type 1, and age and the number of UTIs in the previous year for women with DM type 2) in combination with a urinary leukocyte count. This results in a model with sufficient accuracy (AUC > 0.75).

Introduction

Asymptomatic bacteriuria (ASB) is more common in women with diabetes mellitus (DM) compared to women without DM.[1-3] In our recent multicenter study the prevalence of ASB among 636 women with DM type 1 or type 2 was four times higher than among women without DM.[4] Despite this high prevalence, the need for screening for and treatment of ASB in these patients is still up for debate.[4,5] The outcome of this discussion depends on the one hand on the potential consequences of ASB and intervention, and on the other hand on the cost-effectiveness of screening and possibilities of implementation.[5] Diabetic women with ASB have an increased risk of developing a symptomatic urinary tract infection (UTI) compared to those without ASB.[6] A recent placebo-controlled study showed that antimicrobial treatment of ASB in women with diabetes did not appear to reduce the incidence of symptomatic UTIs.[7] However, it is too premature to conclude that therefore treatment of ASB is not needed, since the described antimicrobial strategy appeared to be insufficient in keeping the women nonbacteriuric, and no conclusion can be drawn from this study on how ASB effects renal function in patients with DM type 1.[8] In a previous study we described that women with DM type 1 and ASB showed a tendency to a faster decline in renal funcion.[6] Since persons with DM make up the fastest growing group of renal dialysis and transplant recipients, accounting for 35% of all new cases of end-stage renal disease in the United States,[9] research on the pathogenesis and prevention of diabetic nephropathy is very important.

The current standard of diagnosing ASB with a urine culture is valid, but time consuming and expensive. Combining optimal history taking with additional testing for leukocyturia to diagnose ASB seems an attractive alternative, but its diagnostic accuracy has not been explored in women with DM. We determined which components of history taking independently contribute to predicting the presence of ASB in women with DM and whether leukocyturia provides any added diagnostic value. Since earlier studies have shown a difference in risk factors for ASB in patients with DM type 1 versus type 2,[4] all analyses were performed separately for women with DM type 1 or type 2. In addition, we developed a prediction rule that, after validation in other diabetic populations, can become an easily applicable rule to diagnose ASB in everyday clinical practice.

Materials and methods

Patients

Data were obtained from our cohort of women with DM who visited the diabetes outpatient clinics of the University Medical Center Utrecht, two non-university hospitals (Catharina Hospital Eindhoven, Diakonessenhuis Utrecht), and the offices of five general practitioners between October 1996 and April 2001.[4,6] Women were eligible for this study if they were between 18 and 75 years of age and had DM (diagnosed by the treating physician). Women were not included if they were pregnant, had been hospitalized within the previous four months, had symptoms of a UTI or fever, had used antimicrobial drugs within the previous fourteen days, or had a known anatomical or functional abnormality of the urinary tract. Approximately 75% of the eligible women participated and no differences in age, type and duration of DM between the nonparticipating patients and the final study group were present (data not shown).[4,6] Symptoms of a UTI were defined as the presence of complaints of dysuria, frequency, urgency, stranguria and/or abdominal discomfort.

At the initial visit, patients were interviewed using a standardized questionnaire, including age, medication, number of UTIs in the previous year, sexual intercourse within the previous days, the use and type of contraceptives, and the number of pregnancies. Information about their medical history, blood pressure, length, weight, and type, duration and secondary complications of DM were obtained from the hospital files or the files of the general practitioners. Hypertension was defined as a systolic blood pressure above 160 mmHg or a diastolic blood pressure above 90 mmHg. In addition, all patients were instructed by one of the investigators to provide a midstream urine specimen for evaluation of leukocyturia and for urinary culture. Glycosylated hemoglobin (GHb) was also determined at baseline. The study was approved by the Medical Ethical Committee of all hospitals and all the patients gave written informed consent.

Urine

Urine cultures were performed in the laboratories of the participating hospitals, according to the local standard procedures. At the Diakonessenhuis Utrecht all urine samples were plated, using quantitative loops. At the University Medical Center Utrecht and the Catharina Hospital Eindhoven, urine samples were screened first, by a uricult dipslide (Orion Diagnostica, Espoo, Finland) and by a direct preparation (viewed at 400 x magnification). Only if at least 10^5 colony forming units per milliliter (cfu/ml) grew on the dipslide or more than five leukocytes or more than ten microorganisms were seen per high power field (hpf), the urine (stored at 4°C) was plated onto blood agar and MacConkey plates. The results were read after 24 hours. Causative microorganisms were identified using Vitek automated identification System (bioMérieux, 's Hertogenbosch, the Netherlands). In all laboratories, the urine was regarded as contaminated and no results were noted if three or more

different microorganisms grew on the plate in a quantity of more than 10^4 cfu/ml. The presence of at least 10^5 cfu of a urinary tract pathogen per ml in a culture of clean-voided midstream urine obtained from a patient without symptoms of a UTI or fever, was used as the 'gold standard' to diagnose ASB.[10]
Leukocyturia was determined directly from an uncentrifuged midstream urine sample by microscopy (400 x magnification). The mean number of leukocytes per hpf was noted after viewing approximately ten fields. The level of leukocyturia was divided in five groups: none, 1 to 5, 5 to10, 10 to 25, or more than 25 white blood cells (WBC) per hpf.

Statistical analysis

SPSS release 10 for Windows 98 was used as statistical software. The association between each potential diagnostic determinant and the presence of ASB was determined by logistic regression and quantified as odds ratios and 95% confidence intervals (CI). Comparisons between means were carried out with Student's t test, and comparisons between nominal or categorical data with the Chi square test. All determinants with a p value < 0.1 were entered together in a multivariable logistic model to evaluate which were independently associated with the presence of ASB. Thereafter, the variables with a p value < 0.1 were included in a 'reduced' model. As a measure of diagnostic accuracy of this logistic regression equation, the area under the receiver operating characteristic curve (AUC) was calculated. The AUC refers to the ability of the model to separate patients with and without the disease. For all possible pairs of patients with and without the outcome (ASB), the AUC reflects the proportion correctly classified patients. The AUC can range from 0.5 (no discrimination) to 1.0 (perfect discrimination).[11] A value of 0.7 to 0.8 is considered to represent reasonable discrimination, a value of > 0.8 good discrimination. The 'reduced' model was then extended with the leukocyte count to quantify the added value of the test result in predicting the presence or absence of ASB. Differences in diagnostic discriminative value between different models were estimated by comparing the AUCs nonparametrically.[12] Finally, the values of the variables were divided in groups and awarded with points to develop a scoring system. The number of points was based on the odds ratios of the variables in the multivariable analysis. All analyses were performed separately for women with DM type 1 and type 2.

Results

Study Population

Data on urinalysis and urinary culture were available from 465 women, 236 women with DM type 1 and 229 women with DM type 2 (*Table 1*). The overall prevalence of ASB was 15%, 11% in women with DM type 1 and 19% in women with DM type 2. When defined as any leukocyte excretion, the overall prevalence of leukocyturia was 38% (33% and 42% for women with DM type 1 and type 2, respectively), and

when defined as ≥ 5 WBC/hpf, the overall prevalence of leukocyturia was 4% (3% and 5% for women with DM type 1 and type 2, respectively).
For women with DM type 1, the duration of DM and the GHb (both p ≤ 0.1) were

Table 1 Baseline characteristics of women with DM type 1 and type 2 with and without ASB

	DM type 1			DM type 2		
	No ASB (n = 210)	ASB (n = 26)	p value	No ASB (n = 186)	ASB (n = 43)	p value
Age (year)	40.5 ± 13.4	45.0 ± 14.9	.15	58.1 ± 10.5	62.8 ± 10.7	.01
Duration DM (year)	20.1 ± 12.6	26.2 ± 14.9	.05	9.0 ± 7.2	9.9 ± 8.1	.51
BMI (kg/m²)	24.7 ± 3.8	24.4 ± 2.8	.55	30.2 ± 6.0	28.7 ± 5.3	.12
Leukocyturia (any excretion of WBC)	59 (28%)	19 (73%)	<.01	67 (36%)	30 (70%)	<.01
Leukocyturia (≥ 5 WBC/hpf)	3 (1%)	3 (12%)	<.01	5 (3%)	7 (16%)	<.01
No. UTIs previous year	0.7 ± 2.0	1.1 ± 3.0	.51	0.5 ± 1.8	1.5 ± 3.4	.01
No. pregnancies	1.4 ± 1.5	1.5 ± 1.7	.73	2.7 ± 2.0	3.3 ± 2.8	.24
Postmenopausal state	46 (22%)	8 (31%)	.31	138 (74%)	33 (77%)	.73
GHb (%)	8.3 ± 1.4	9.0 ± 1.9	.01	8.4 ± 1.7	8.4 ± 1.8	.84
Sexual intercourse	82 (39%)	11 (42%)	.75	48 (26%)	11 (26%)	.98
Hypertension	24 (11%)	5 (19%)	.25	54 (29%)	17 (40%)	.18

Continuous variables are expressed as means ± standard deviations, categorical variables as numbers (and percentages). DM indicates diabetes mellitus; ASB asymptomatic bacteriuria; UTIs urinary tract infections; BMI body mass index; WBC white blood cells; hpf high power field; GHb glycosylated hemoglobin.

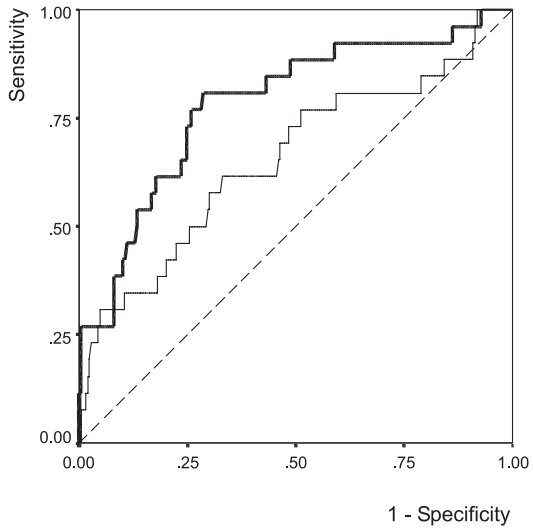
selected for multivariable analysis (*Table 2*). The adjusted odds ratios of the duration of DM and the GHb were 1.03 (95% confidence interval (CI) 1.00–1.06) and 1.35 (95% CI 1.04–1.75), respectively. The AUC of the model including these variables was 0.66 (95% CI 0.53–0.78). After addition of leukocyturia, the AUC increased considerably to 0.78 (95% CI 0.68–0.88) (*Figure 1*). This increase in diagnostic accuracy was statistically significant (p = 0.02).

Table 2 Predictors of asymptomatic bacteriuria in women with DM type 1

	Unadjusted odds ratio (95% CI) [1]	Adjusted odds ratio (95% CI)	
		Reduced model	Reduced model plus leukocyturia
Age (year)	1.02 (0.99–1.05)	Not included	Not included
Duration of DM (year)	1.03 (1.00–1.06)	1.03 (1.00–1.06)	1.03 (1.00–1.06)
GHb (%)	1.37 (1.06–1.77)	1.35 (1.04–1.75)	1.24 (0.95–1.62)
Leukocyturia (0 to 4)[2]	3.38 (1.83–6.26)	Not included	3.02 (1.63–5.61)

[1] All variables with p ≤ 0.1 were selected for multivariable regression to calculate the adjusted odd ratios.
[2] The number of leukocytes is divided in five groups (see methods).
CI indicates confidence interval; DM diabetes mellitus; GHb glycosylated hemoglobin.

Figure 1



Receiver operating characteristic curves of the model for women with diabetes mellitus type 1. The area under the curve (AUC) of the model without leukocyturia (thin line) is 0.66; the AUC with leukocyturia (thick line) is 0.78.

For women with DM type 2, the patients' age and the number of symptomatic UTIs in the previous year were included in the multivariable analysis (*Table 3*); the adjusted odds ratios were 1.05 (1.01–1.09) and 1.24 (1.05–1.47), respectively. The AUC of this was 0.70 (95% CI 0.61–0.80). Adding leukocyturia resulted in a statistically significant increase (p = 0.02) of the AUC to 0.79 (95% CI 0.71–0.86) (*Figure 2*).

Table 3 Predictors of asymptomatic bacteriuria in women with DM type 2

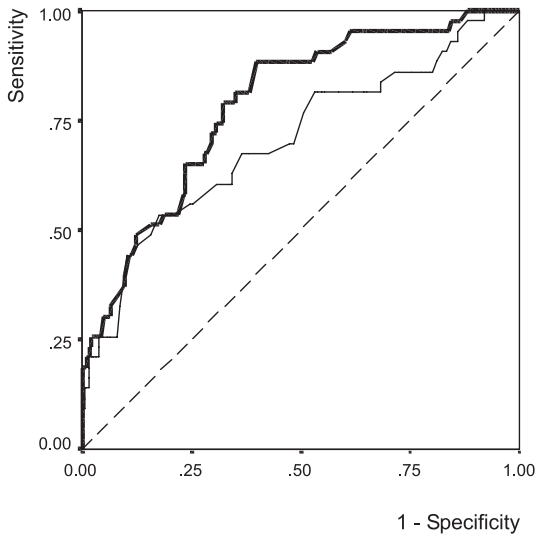
	Unadjusted odds ratio (95% CI) [1]	Adjusted odds ratio (95% CI)	
		Reduced model	Reduced model plus leukocyturia
Age (year)	1.05 (1.01–1.09)	1.05 (1.01–1.09)	1.04 (1.00–1.08)
No. UTIs previous year	1.24 (1.05–1.46)	1.24 (1.05–1.47)	1.20 (1.00–1.43)
Leukocyturia (0 to 4)[2]	2.26 (1.52–3.56)	Not included	1.99 (1.33–2.99)

[1] All variables with p ≤ 0.1 were selected for multivariable regression to calculate the adjusted odd ratios.

[2] The number of leukocytes is divided in five groups (see methods).

CI indicates confidence interval; DM diabetes mellitus; UTIs urinary tract infections.

Figure 2



Receiver operating characteristic curves of the model for women with diabetes mellitus type 2. The area under the curve (AUC) of the model without leukocyturia (thin line) is 0.70; the AUC with leukocyturia (thick line) is 0.79.

Scoring System

Having the results mentioned above, it is possible to develop a model that can be used in clinical practice. Scoring the values of the selected variables based on the odds ratios resulted in two diagnostic rules shown in *Table 4*. Using these rules we can predict the probability of ASB in a given patient. Each value of these rules (i.e., the test is 'positive' if the total number of points is above this chosen cut-off value) has its own sensitivity and specificity. To use this model with both a sensitivity and a specificity of 85% would mean that women with DM type 1 with a score ≥ 7 are predicted to have and with a score ≤ 4 are predicted not to have ASB. A score between these values is non-conclusive and must be followed by a urine culture to diagnose ASB. For women with DM type 2, the same criteria are met by cut-off values of respectively ≥ 9 and ≤ 7. Using these values, 76% and 73% of all urine cultures could be omitted in women with DM type 1 and type 2, respectively.

Table 4 Prediction rules for diagnosing asymptomatic bacteriuria in women with DM type 1 or type 2

DM type 1			DM type 2		
Variable	**Value**	**Points**	**Variable**	**Value**	**Points**
Duration DM (year)	< 10	0	**Age (year)**	< 30	0
	10 to 19	1		30 to 39	1.5
	20 to 29	2		40 to 49	3
	30 to 39	3		50 to 59	4.5
	40 to 49	4		60 to 69	6
	≥ 50	5		≥ 70	7.5
GHb (%)	< 8	0	**No. UTIs previous year**	0	0
	8 to 8.9	1		1	2
	9 to 9.9	2		2	4
	10 to 10.9	3		≥ 3	6
	11 to 11.9	4			
	≥ 12	5			
Leukocytes / hpf	None	0	**Leukocytes / hpf**	None	0
	1 to 5	3		1 to 5	2.5
	5 to 10	6		5 to 10	5
	10 to 25	9		10 to 25	7.5
	> 25	12		> 25	10

DM indicates diabetes mellitus; GHb glycosylated hemoglobin; UTIs urinary tract infections; hpf high power field.

Discussion

We have described that the combination of three easily obtainable items provides a diagnostic tool with sufficient accuracy to diagnose ASB in women with DM type 1 and type 2. As expected from our previous studies on the risk factors of ASB, and from the difference in etiology of the two types of diabetes, the contributing variables were different for women with DM type 1 and women with DM type 2.
A scoring system based on these items as a screening tool could save the costs and time of a urine culture in a proportion of patients. Using 85% as the criterion of sensitivity and specificity, 76% and 73% of all urine cultures could be omitted in women with DM type 1 and type 2, respectively. However, other criteria can be desired in which case other cut-off values have to be applied. For example, to achieve a high specificity of ≥ 96%, a high score (≥ 9 for women with DM type 1 and ≥ 13 for women with DM type 2) is needed to diagnose ASB. If follow-up studies of diabetic women would show an association between ASB and renal function deterioration, as suggested by our preliminary data,[6] it would be important to diagnose all cases of ASB and to have a low number of false negative test results. In that case, a high sensitivity, and thus a low threshold value, would be more important. So it seams clear that further follow-up studies on the consequences of

ASB in this specific patient group are required to provide the crucial information to determine the optimal test criteria.

Recently, Bent et al. studied the accuracy of history taking and physical examination for the diagnosis of an acute uncomplicated UTI in nondiabetic women presenting with one or more symptoms of a UTI.[13] In their systemic review they show that dysuria, frequency, hematuria, back pain and costovertebral angle tenderness significantly increase the probability of UTI. They conclude that specific combinations of symptoms can increase the likelihood of UTI to more than 90%, effectively diagnosing UTI based on history alone. For the added value of leukocyturia, the authors refer to a review of Hurlbut and Littenberg who studied the value of the dipstick urinalysis (nitrite and leukocyte esterase test).[14] Due to the high pre-test probability (approximately 50%) of a patient presenting with one or more symptoms of UTI, neither history taking, physical examinations nor dipstick analysis were able to rule out symptomatic UTI.[13] In all reviewed studies, both the patient population (nondiabetic women) and the study outcome (symptomatic UTI) were different from our study, which explains the difference in clinical predictors. One of the studies reviewed is a clinical study by Wigton et al.[15] They developed a decision rule to predict the presence of a positive urine culture in 216, mostly nondiabetic women suspected of having a (symptomatic) UTI. Retrospectively, five variables (history of previous UTI, back pain, microscopic pyuria, hematuria and bacteriuria) were selected for the decision rule. The AUC of the model was 0.78. Using this model with a sensitivity value of 88%, a urine culture could be avoided in 24% (score four to five points). Microscopic pyuria was found to be the strongest predictor of infection.

In our study, adding microscopic leukocyturia to the model significantly increased the AUC, both in DM type 1 and type 2, meaning that it contributed to a higher accuracy in diagnosing ASB. Although a leukocyte count by a chamber count technique is more reliable,[16] counting leukocytes per hpf is still the most widely practiced method. Stamm et al. showed that a leukocyte count of ten per cubic millimeter in otherwise healthy women is indicative of a symptomatic UTI. However, we found fewer leukocytes in the urine of diabetic women with ASB compared to nondiabetic women with ASB, probably due to the fact that urinary cytokine secretion is impaired in women with DM.[17] In addition to our study, it would be interesting to see whether a model with a leukocyte count determined by a leukocyte esterase dipstick provides comparable results.

Several limitations of our study need to be addressed. We defined ASB using one urine culture. ASB is generally defined as the presence of at least 10^5 cfu/ml of the same single species in two consecutive cultures of clean-voided specimens of midstream urine from an individual without symptoms of a UTI or fever.[18] Since the cut-off point of 10^5 cfu/ml seems the most important to discriminate between true bacteriuria and contamination,[19] the need for the second urine culture is not clear.[20] New molecular methods have enabled us to identify different strains and showed that nearly half the patients previously classified as having ASB based on the presence of 'the same' microorganism in two consecutive cultures, actually had

different *E. coli* strains in these cultures.[20] It is worthwhile to mention that several recent studies on ASB use only one culture in the definition of ASB, whereas in others only the positive cultures are repeated.[20] Finally, our aim was to efficiently diagnose ASB, and not to identify the infecting agent or the antibiotic sensitivity. However, in the diagnostic work-up for ASB, this will rarely be required.

A scoring method as described here has the highest value in the population from which it is derived. To become an easily applicable decision tool in daily patient care it is important to validate the rule in other diabetic populations. We have made effort to include a representative sample of the diabetic population, by asking all eligible diabetic women to participate who came for their regular visit either to the diabetes outpatient clinics of three hospitals, among which both a university and two non-university hospitals, or the offices of general practitioners. Therefore, we assume that our prediction rule will prove to be applicable for widespread use.

In conclusion, two variables routinely obtained in clinical practice (duration of DM and GHb for women with DM type 1, and age and the number of UTIs in the previous year for women with DM type 2) in combination with a urinary leukocyte count accurately predict the presence of ASB. By using our diagnostic rule, urine culture is no longer necessary in a substantial part of women with DM.

Acknowledgements

This study was supported by a grant from the Dutch Diabetes Fund (project number 95.123).

We thank the following physicians: Th.J. Collet, E.W.M.T. ter Braak, M.C. Castro Cabezas, T.W. van Haeften, J.B.L. Hoekstra, P.C. Ligtenberg-Oldenburg, H.W. de Valk, H.E. Westerveld, P.M.J. Zelissen (University Medical Center Utrecht), B. Bravenboer (Catharina Hospital Eindhoven) J.B.L. Hoekstra, W.M.N. Hustinx (Diakonessenhuis Utrecht), the students M. Bellaar, M. Kuipers, and R. Jansen who helped recruit the patients, and the medical microbiologists J. Verhoef (University Medical Center Utrecht), A.R. Jansz (St. Joseph Hospital, Veldhoven), and R.J.A. Diepersloot (Diakonessenhuis, Utrecht).

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Seven

Development of a quantitative real-time PCR assay for the detection of *Escherichia coli* in urine samples

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Submitted for publication

Abstract

Introduction

The aim of this study was to develop a real-time Polymerase Chain Reaction (PCR) for the rapid detection of *Escherichia coli* bacteriuria.

Methods

PCR primers and probe specific for *E. coli* were designed for the assay. Fifty *E. coli* strains and 41 non-*E. coli* strains were tested, including many uropathogens and members of the vaginal and anal flora. For clinical evaluation, 42 clinical urine specimens were tested; the results were compared to those of conventional cultures.

Results

The lower detection limit was 10^4 cfu of *E. coli* per ml of urine. The laboratory sensitivity and specificity of the real-time PCR were 100% (50/50) and 98% (40/41), respectively. Testing the clinical urine specimens, the sensitivity and specificity of the real-time PCR were 92% (11/12) and 87% (26/30), respectively. The assay required three hours.

Conclusion

This assay provides a new tool in diagnosing *E. coli* bacteriuria in fresh and stored samples.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections acquired by humans, with a lifetime risk among women of approximately 60%.[1] *Escherichia coli* causes more than 80% of uncomplicated infections, as well as the majority of recurrent and complicated UTIs.[2-4] The urine culture is the golden standard to diagnose an *E. coli* UTI.[5] However, culture methods have some disadvantages. Culturing requires at least 24 hours to yield results, and is only useful in freshly voided urine. Especially in research setting, it can be desirable to store human samples like urine, in order to collect and test them when appropriate.

In the last few years the real-time Polymerase Chain Reaction (PCR), a more quantitative nucleic acid amplification technique than the regular PCR, has been used more frequently in clinical and research settings. Its advantages include saving time, while being highly sensitive and specific. Its disadvantage is that sensitivity testing cannot be performed. One of the glutamate decarboxylase (gad) genes, *gadA*, has been used for identification of *E. coli* by PCR amplification before,[6] but none of the tests previously described based on the real-time PCR technique were designed to detect *E. coli* in urine.

The aim of the present study was to develop a real-time PCR-based assay for the rapid detection of *E. coli* bacteriuria, both in fresh and in stored urine specimens. We first determined the sensitivity and specificity of the test with *E. coli* and non-*E. coli* strains, including the most prevalent uropathogens and many members of the vaginal and anal flora, suspended in sterile water. After that, clinical urine specimens were tested and the results were compared to those of a conventional urine culture.

Materials and Methods

Microorganisms

A total of 50 *E. coli* strains, all human clinical isolates, were used in this study. The strains were isolated from urine of patients with a complicated or an uncomplicated UTI, or with asymptomatic bacteriuria. The identification of the bacteria was determined by the Vitek automated identification system (bioMérieux, Den Bosch, the Netherlands). All strains were cultured on blood agar plates at 37°C under aerobic conditions and suspended in sterile water-for-injection. The bacterial concentration was calculated by the optical density at 660 nm (Dr Lange Photometer, Berlin, Germany). This optical density corresponded with a certain bacterial concentration, determined by plating serial dilutions of bacteria on blood agar plates (calculation according to Watson). Hundred μL of each bacterial suspension was heated for two minutes at 1000 Watt in a microwave oven to prepare the DNA template.[7] (Five μl of this suspension was added to the reaction volume, see further.)

A wide variety of Gram positive and Gram negative bacterial strains, including the most prevalent uropathogens and many members of the vaginal and anal flora

were used to test the specificity of the PCR assays (total number 41, see *Table 1*). The non-*E. coli* strains also included bacterial species that are phylogenetically close to *E. coli*, for example *Klebsiella pneumoniae*. In addition, *Shigella flexneri* was included because the primers used for the real-time PCR showed comparable homology to this species as to *E. coli* in the BLAST search of GenBank (see further). Amplifications from standardized bacterial suspensions were performed to quantify the bacterial concentration of the different samples. All strains were identified by the Vitek automated identification system and grown on media under conditions that support their optimal growth. The bacteria were subsequently prepared as described above for *E. coli*. The presence of bacterial DNA was confirmed by a conventional PCR specific for the conserved regions of the 16S rRNA gene as described before.[8]

Table 1 Bacterial strains used to test the specificity of the *E. coli* real-time Polymerase Chain Reaction

Gram positive bacteria	Gram negative bacteria
coagulase negative staphylococcus (n = 2)	*Acinetobacter calcoaceticus* (n = 2)
Corynebacterium sp. (n = 1)	*Aeromonas hydrophilia* (n = 1)
Enterococcus faecalis (n = 3)	*Alcaligenes faecalis* (n = 1)
Group B streptococcus (n = 7)	*Citrobacter freundii* (n = 2)
Lactobacillus arabinosis (n = 1)	*Enterobacter cloacae* (n = 5)
Lactobacillus leichmanii (n = 1)	*Klebsiella oxytoca* (n = 2)
Staphylococcus aureus (n = 2)	*Klebsiella pneumoniae* (n = 2)
Staphylococcus epidermidis (n = 1)	*Proteus mirabilis* (n = 1)
Staphylococcus saprophyticus (n = 1)	*Proteus vulgaris* (n = 2)
viridans streptococcus (n = 1)	*Pseudomonas* sp. (n = 1)
	Serratia odorifera (n = 1)
	Shigella flexneri (n = 1)

Oligonucleotides

The *gadA* gene of *E. coli* was used to identify regions conserved in *E. coli* only.[6] Primers and probe complementary to these conserved regions were chosen using the Taqman Probe and Primers Design software. A BLAST search of GenBank was performed to study the specificity. Primers were synthesized and purified by Isogen Bioscience BV (Maarsen, the Netherlands). The adjacent probe (Applied Biosystems, Warrington, United Kingdom) contained a fluorescent reporter, dye 6-carboxyfluorescein (FAM), covalently linked to the 5' end of the oligonucleotide. This allows fluorescence resonance energy transfer to liberate an increased fluorescence signal after replacement from their target sequences.

Real-time PCR assay

Real-time PCRs were performed in MicroAmp Optical 96-well reaction plates with Optical Caps (PE Biosystems) by use of the ABI PRISM 7700 Sequence Detection System (PE Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Each 25 µl reaction volume consisted of 12.5 µl 2x Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) that contains AmpliTaq Gold DNA polymerase, 300 nM forward primer (5'-ACCGACATCGTGGTGATGC-3'), 300 nM reverse primer (5'-AGCAACAGTTCAGCAAAGTCCA-3'), and 175 nM probe (5'-CATTATGTGTCGTCGCGGCTTCGAA-3'); 5 µl of DNA template was the last ingredient added. Every sample was analyzed in duplicate. Cycling parameters were first the uracil-*N*-glycosylase (UNG) reaction at 50°C for two minutes, then AmpliTaq Gold activation at 95°C for ten minutes, followed by 40 cycles of denaturation at 95°C for fifteen seconds - combined annealing and extension at 60°C for one minute. Emitted fluorescence from each well was measured during both the denaturation and annealing/extension steps in every cycle. Amplification plots were constructed using the ABI PRISM 7700 Sequence Detection System software, version 1.7 (PE Biosystems).

Control reactions to which no DNA was added (no template control) were performed routinely to verify the absence of DNA carryover. *E. coli* strain Ctrl 39, a well known clinical strain,[9] was used as positive control. Concomitant amplification of the positive control (in a separate reaction well) allowed verification of the efficiency of the PCR to ensure the absence of inhibition by the PCR reagents.

Clinical specimen

Midstream urine samples were collected from 42 women visiting the outpatient department of internal medicine at one university hospital (University Medical Center Utrecht) and three non-university hospitals (Diakonessenhuis Utrecht, Jeroen Bosch Hospital 's Hertogenbosch, Catharina Hospital Eindhoven).[10] Upon receipt, part of the urine sample was cultured at the local laboratories according to standard procedures, as described before.[8] The identification of the bacteria was determined by the Vitek automated identification system. Culture results were noted as: negative (i.e., no growth of any uropathogen $\geq 10^4$ cfu/ml), or positive (i.e. growth of one or two uropathogens $\geq 10^4$ cfu/ml). Contaminated urine was defined as the growth of at least three different microorganisms in one urine specimen. The identification of these microorganisms was not further performed.

The remaining urine was stored at –20°C until further use. One ml of urine was centrifuged at 16,250 x g for 5 minutes. The pellet was washed twice, suspended in 1 ml of sterile injection water, and heated for two minutes at 1,000 Watt in a microwave oven. Finally, 5 µl was added to the real-time PCR reaction volume, and the PCR was performed as described above.

Results

Evaluation of the *E. coli*-specific real-time PCR

From the panel of Gram positive and Gram negative uropathogenic species suspended in sterile water only *E. coli* could be detected by the production of an increased fluorescence signal that was interpreted as a positive PCR result. The fluorescence resonance energy transfer signal for the positive control performed in a separate well was detected for all PCR reactions, thereby showing the absence of significant PCR inhibition.

The real-time PCR assay was able to efficiently detect all 50 *E. coli* strains used in this study, showing a perfect correlation with standard culture-based identification methods. Therefore the sensitivity of the real-time PCR was 100% (50/50). All tested uropathogens and members of the vaginal or anal flora that were included in this study gave a negative test result. *Shigella flexneri* however showed a positive test result, as expected. So the specificity of the real-time PCR was 98% (40/41).

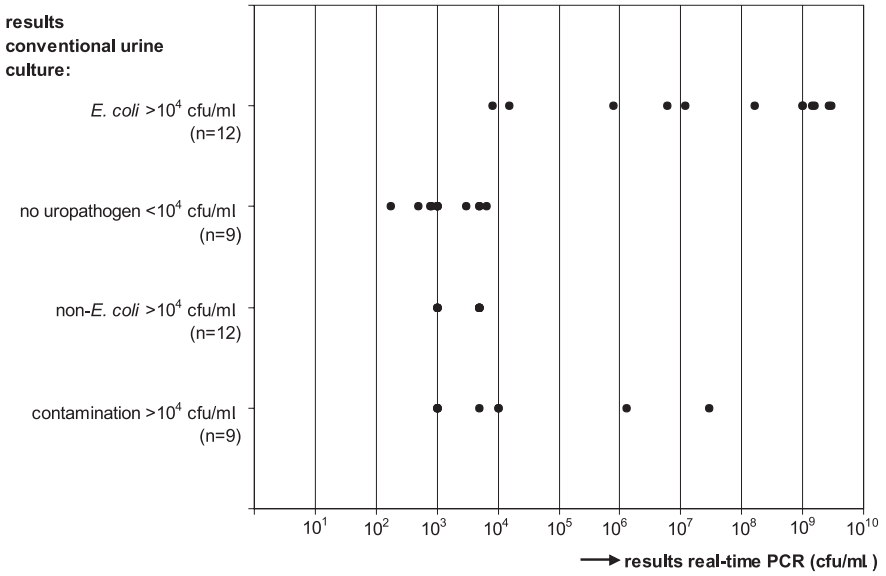
Identification of *E. coli* bacteriuria in women

We tested 42 clinical urine samples from female patients visiting the outpatient department. These women included asymptomatic women with and without bacteriuria, women with cystitis, and women with pyelonephritis. As determined by our local standard culture methods twelve of the urine samples were positive for *E. coli* (two of those samples also grew *Proteus mirabilis* and Group B *streptococcus*, respectively). Twelve samples were positive for one or two non-*E. coli* strains (including amongst others *Candida albicans*), nine showed no growth, and nine were 'contaminated' (from these urine samples three or more pathogens were isolated; all these samples showed a colony count $\geq 10^4$ cfu/ml).

The results are summarized in *Figure 1*. With a positive PCR result defined as a colony count of $\geq 10^4$ per ml, the sensitivity of the test was 92% (11/12; 95% confidence intervals (CI) 0.62–1.00). Including the contaminated cultures, the specificity was 87% (26/30; 95% CI 0.68–0.96); excluding the contaminated urine samples, the specificity was 100% (21/21; 95% CI 0.81–1.00) (see discussion). The positive and negative predictive value of the test were 73% (11/15; 95% CI 0.45–0.91) and 96% (26/27; 95% CI 0.79–1.00), respectively. The cut-off value can easily be adapted to a higher value, for example 10^5 cfu/ml. In our case this would have resulted in a sensitivity of 100% (10/10; 95% CI 0.66–0.99) and a specificity of 94% (30/32; 95% CI 0.78–0.99) (*Table 2*).

In clinical urine samples, the lower detection limit (i.e., minimal cfu per ml of urine that can be detected) of the real-time PCR was 10^4 cfu per ml of urine. Lower bacterial counts could not be quantified reliable due to non-specific background amplification. As described earlier,[11,12] this was caused by amplification of the *E. coli*-derived polymerases in the Taqman Universal PCR Master Mix, and possible also by large amounts of non-*E. coli* strains that are present in the urine, which can give aspecific background signals. The no template controls showed a signal

Figure 1



Results generated by the real-time Polymerase Chain Reaction (PCR), in a logarithmic scale, correlated to conventional culture results of 42 clinical urine samples. The non-*E. coli* strains include *Proteus mirabilis*, Group B streptococcus, *Staphylococcus aureus*, *Aeromonas* sp., *Enterococcus faecalis*, *Acinetobacter lwoffi*, coagulase negative staphylococcus, *Klebsiella pneumoniae, Enterobacter cloacae*, *Lactobacillus*, *Corynabacterium* sp. and *Candida* sp. For the definition of contamination see text. Cfu indicates colony forming unit.

Table 2 Comparison of the real-time PCR with conventional urine cultures.[a]

	10^4 cfu/ml[b] (95% CI)		10^5 cfu/ml (95% CI)	
sensitivity	0.92	(0.60–1.00)	1.00	(0.66–1,00)
specificity	0.87	(0.68–0.96)	0.94	(0.78–0.99)
positive predictive value	0.73	(0.45–0.91)	0.83	(0.51–0.97)
negative predictive value	0.96	(0.79–1.00)	1.00	(0.86–1.00)

[a] Contaminated urine samples are included in the analyses and considered negative when tested by routine culturing (see text); [b] The bacterial count above which level the urine sample was defined as positive by routine culturing ("the gold standard"); CI indicates confidence interval; PCR Polymerase Chain Reaction.

comparable to maximally 10^3 cfu of *E. coli* per ml of urine. Therefore a lower detection limit would result in a lower specificity and therefore more false-positive results.

For the standard urine culture the time required for identification and quantification of bacteriuria is at least 24 hours. In comparison, the time required for the real-time PCR from urine was approximately three hours, including the computer-based data analysis.

Discussion

In addition to previous studies on species-specific as well as on broad range detection of bacterial DNA,[12] we presented a real-time PCR for the quantitative detection of *E. coli* bacteriuria and validated this in true clinical urine specimens. Especially in research setting, this assay provides a new tool in diagnosing *E. coli* bacteriuria. The standard urine cultures, nitrite test, and the different tests for leukocyturia require fresh urine specimens, while the real-time PCR can also be used in old and stored urine samples. In specific cases, for instance in epidemiological studies in which the significance of previous bacteriuria for different diseases is studied, this can be a welcome supplement.

To be used in clinical practice, the real-time PCR has both advantages and disadvantages when compared to urine culture and other tests like the leukocyte esterase and the nitrite test. The current standard to diagnose true bacteriuria is defined by the prevalence of 10^5 cfu of a uropathogen per ml urine determined by a urine culture. A urine culture is time consuming, varying from a minimum of 24 hours in case of a negative culture, to approximately 48 hours when positive. Consequently, in daily clinical practice most physicians rely on the positive predictive value of clinical symptoms such as dysuria and frequency. However, depending on the presence of some, and the absence of other symptoms (like vaginal discharge) only in 50 to 90% of dysuric women can a uropathogen be isolated from the urine, whereas in the remaining women another diagnosis should be considered.[13,14] Other diagnostic tests more rapid than the standard culture include the leukocyte esterase test and the nitrite test. However, a recent meta-analysis again showed the great variety in sensitivity and specificity of these tests, even when combining the leukocyte esterase test with the nitrite test (sensitivity 45 to 92% en specificity 62 to 87%).[15] Moreover, in subjects with asymptomatic bacteriuria, pyuria is less frequent than in patients with acute cystitis (for example, in young women with asymptomatic bacteriuria, the prevalence of pyuria is approximately 32%).[16] Therefore, relying on these tests or on symptoms only, may lead to under- and overtreatment with (broad-spectrum) antibiotics, whereas the latter can not only lead to unnecessary side effects but is also associated with the increasing antimicrobial resistance among uropathogens.[17] A rapid quantitative test with a high sensitivity and specificity for diagnosing *E. coli* bacteriuria would allow for more efficient diagnosis and treatment.

The results of the real-time PCR differ from those of the standard culture when examining contaminated urine samples (the latter defined as the presence of three or more uropathogens). We found different specificity outcomes of our assay, depending on whether or not we included contaminated samples in the calculations. This difference was to be expected: "contamination" with *E. coli* strains will be detected by the real-time PCR technique, but ignored with the conventional culture if the limit of three microorganisms is exceeded. Since the clinical relevance of these results is not known, it depends on the situation whether this is an advantage or a disadvantage of the real-time PCR.

Our study has some limitations. Due to the species-specific nature of our test, other uropathogens, which can be detected by routine culturing, are missed with this real-time PCR. Therefore, it can only be applied in uncomplicated UTIs of which the large majority is caused by *E. coli*. In addition this test can be used in research in which the outcome of interest is *E. coli* bacteriuria. However, in complicated UTIs one must consider the appearance of a broader variety in uropathogens. Another disadvantage of the real-time PCR is that sensitivity testing cannot be performed.
The lowest detection limit of the real-time PCR was 10^4 cfu per ml of urine, which is the generally accepted level above which coliform bacteriuria is considered clinically relevant. Lower bacterial counts could not be quantified reliable due to background amplification of the *E. coli*-derived polymerases in the Taqman Universal PCR Master Mix, as described earlier,[11,12] and possible also by non-specific amplification by large amounts of non-*E. coli* strains when present in the urine. We did not experience any effect on the sensitivity of the assay caused by the human DNA present in voided urine samples. The specificity of the test was not 100%. Of all the non-*E. coli* strains tested, only *Shigella flexneri* gave a positive signal in the test. Although this species has been described to cause diarrhea occasionally, it is not a normal inhabitant of the human gut, neither a member of the normal vaginal flora or a uropathogen. Therefore, it is not considered clinically relevant when testing urine samples.
Most clinical laboratories do not have a real-time PCR available yet. Implementation of this technique therefore will currently not be cost-effective when comparing it with routine urine culturing.
Our assay is developed for *E. coli*, the most prevalent uropathogen. A promising development and goal for future studies will be the development of real-time PCR assays that detect not only other uropathogens but also virulence factors, antimicrobial resistance and gene mutations that are associated with antimicrobial resistance.

Conclusions

We developed a quantitative real-time PCR assay for the detection of *E. coli* in urine samples. The high sensitivity and specificity in addition to the short time required for the test makes this real-time PCR a new tool in diagnosing *E. coli* bacteriuria. In contrast to conventional methods, the technique is suited for quantifying *E. coli* in stored samples.

Acknowledgement

This program has been supported by the Foundation "De Drie lichten", the Netherlands.

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Eight

Escherichia coli bacteriuria in female adults is not associated with a decline in renal function at long-term follow-up

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Submitted for publication

Abstract

Introduction

To investigate whether *Escherichia coli* bacteriuria is associated with a decline in renal function or end-stage renal failure after a long-term follow-up.

Methods

To address the relation between *E. coli* bacteriuria and renal function development, we performed a full cohort analysis for women who participated in two population based studies. Between 1974 and 1986 all women, aged 39 to 68 years, who lived in the city of Utrecht, the Netherlands, were invited to participate in a breast-cancer-screening program. This baseline cohort consisted of 38,994 women who completed a questionnaire, underwent a medical examination, and collected a midstream morning urine sample which was stored since. *E. coli* bacteriuria was diagnosed from these urine samples by a real-time Polymerase Chain Reaction. From 1993 to 1997 another study was performed in the same area with 17,357 participants, of whom a blood sample was drawn. The Cockcroft-Gault formula was used to estimate the creatinine clearance. In the cohort analysis 490 women were included with a mean follow-up of 11.5 ± 1.7 years. To examine the relation with end-stage renal failure, a nested case-control study was performed; cases were all women who underwent kidney-replacing therapy in the Netherlands between participation in the baseline cohort and May 2002 (n = 49, mean duration from baseline until kidney-replacing therapy of 13.8 ± 7.4 years).

Results

The mean age at baseline was 45.0 ± 3.2 years. Forty-eight women (10%) had *E. coli* bacteriuria at baseline. After 11.5 years the mean creatinine clearance for women with versus without bacteriuria was 87 ± 21 and 85 ± 18 ml per minute, respectively. *E. coli* bacteriuria at baseline was not associated with creatinine levels, adjusted for age and weight (p = 0.71). In the case-control analysis the prevalence of *E. coli* bacteriuria was 14% among both cases and the controls. The odds ratio for the development of renal failure in the presence of *E. coli* bacteriuria at baseline, corrected for age, was 1.1 (95% confidence interval 0.4–2.8, p = 0.86).

Conclusion

E. coli bacteriuria is not associated with a decline in renal function or the development of end-stage renal failure in a population of generally healthy adult women during 12 to 14 years of follow-up.

Introduction

Chronic kidney disease is an increasing public health problem. In the United States, the prevalence is estimated to be approximately 11% of the adult population.[1] Chronic kidney disease may progress to end-stage renal failure, a condition associated with high morbidity and mortality. In the Netherlands and the United States, the prevalence rates of end-stage renal failure are 65.8 and 144.6 per 100,000 population, respectively.[2,3]

Although the urinary tract is normally sterile, (asymptomatic) bacteriuria is a common phenomenon, especially in women. Different studies report a prevalence of approximately 5% among healthy young women, increasing to over 20% in the elderly.[4,5] *Escherichia coli* is the most prevalent causative microorganism in both symptomatic and asymptomatic bacteriuria, accounting for more than 80% of uncomplicated urinary tract infections (UTIs).[6] The large majority of uropathogenic *E. coli* possess type 1 fimbriae that have shown to cause renal scarring in animal studies.[7] Previous studies have demonstrated that patients with renal scarring due to pyelonephritis are at increased risk for the development of hypertension and chronic kidney disease.[8] For (asymptomatic) bacteriuria this relation is less clear.[9,10] In a previous study we reported that bacteriuria might influence renal function of women with diabetes type 1.[11]

The present study aims to address the question whether *E. coli* bacteriuria is associated with an increased risk of the development of chronic kidney disease or end-stage renal failure during a long-term follow-up period.

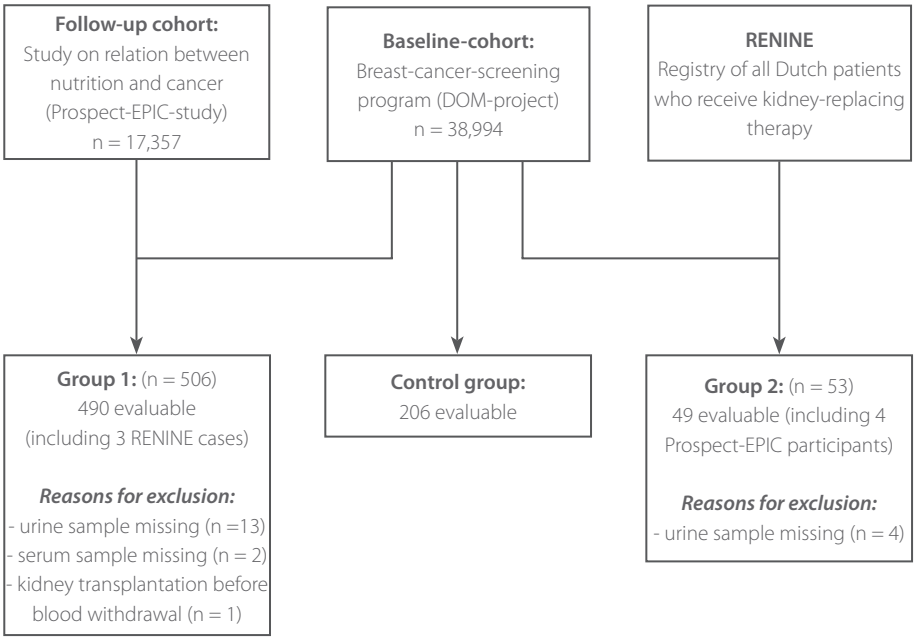
Methods

Study population

To address the relation between *E. coli* bacteriuria and renal function development, we performed a full cohort analysis for women who participated in two population based studies (*Figure 1*). Between 1974 and 1986 all women, born between 1911 and 1945, who lived in the city of Utrecht and surroundings, the Netherlands, were invited for a breast-cancer-screening program, with a participation rate of 68 to 72%.[12] A total number of 38,994 women, aged 39 to 68 years old at intake, participated (the baseline cohort). Baseline measurements, performed between 1974 and 1986, included extensive questionnaires, a short medical examination, and the collection of a midstream morning urine sample. Data obtained through the questionnaires included age, marital status, parity, menopausal age, diet and drug use. During the medical examination weight and height were measured. Approximately 200 ml urine was stored in plastic polypropylene jars, without preserving agents, and stored at –20°C for future analyses.[13] All women gave oral consent to use their data and urine samples for future scientific research.

From 1993 to 1997, participants in the breast-cancer-screening program received an invitation by mail to join an additional study to assess the relation between nutrition and cancer and other chronic diseases, the Prospect-EPIC study (the

Figure 1



follow-up cohort). A total of 17,357 women living in Utrecht and surroundings agreed to take part (participation rate 34.5%).[14] Participants were between 49 and 70 years old at enrolment. Information was collected on the basis of two self-administered questionnaires and a medical examination including blood pressure. Non-fasting blood samples were successfully drawn from 97.5% of the women, and stored under liquid nitrogen at –196°C. Approximately 88% of the women signed a detailed informed consent, enabling the researchers to use their blood samples for future analysis, and to obtain information on future morbidity and mortality.[14]

A total number of 506 women participated in both the baseline cohort and the follow-up cohort. Sixteen women had to be excluded for the following reasons: a missing urine sample (n = 13), a missing serum sample (n = 2), or a kidney transplantation before blood withdrawal (n = 1). Finally, 490 women were included in the prospective study to assess the relation between bacteriuria and renal function. The mean duration of follow-up was 11.5 ± 1.7 years, ranging from 8.1 to 18.6 years from baseline until participation in the follow-up study.

To obtain follow-up information on end-stage renal failure, we obtained data from the Renal Replacement Registry Netherlands (RENINE) that were available May 2002. RENINE is a foundation in which all Dutch nephrologists participate and where patients are registered who at one time have used kidney replacing therapy (hemodialysis or renal transplantation), with a coverage rate throughout the years of nearly 100%.[2] Data from the baseline cohort and RENINE were matched on

(maiden and married) name combined with date of birth to select the cases (*Figure 1*). A group consisting of four times the number of cases was randomly selected from the baseline cohort to form the control group. Four women participated in the follow-up cohort and were also selected as one of the cases who received kidney replacing therapy during follow-up; as mentioned above one woman underwent kidney transplantation before blood withdrawal (and was excluded for the cohort analysis), three women developed end-stage renal failure thereafter (and were included in both analyses). After excluding four individuals with a missing urine sample 49 cases and 206 controls were included. Among the cases, the mean duration until the date of kidney replacing therapy was 13.8 ± 7.4 years, with a minimum and maximum duration of 1.6 and 25.5 years, respectively. In the control group, the mean follow-up (i.e. the time from participation in the baseline cohort until study-endpoint in May 2002) was 27.0 ± 0.2 years.
This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands.

E. coli bacteriuria

E. coli bacteriuria was defined as the presence of 10^5 colony forming units (cfu) of *E. coli* per ml of urine. It was diagnosed by a real-time Polymerase Chain Reaction (PCR) that we developed and validated beforehand (*see Chapter 7*), a technique that was also used by others.[15,16] PCR primers and probe complementary to regions of the gadA gene specific for *E. coli* were designed for the real-time PCR assay. The laboratory sensitivity and specificity of the real-time PCR tested with 50 *E. coli* strains and with 41 non-*E. coli* strains (including the most prevalent uropathogens and many members of the vaginal and anal flora) were 100% (50/50) and 98% (40/41), respectively. For clinical evaluation, 42 clinical urine specimens (12 with and 30 without *E. coli*) were tested and the results were compared to those of a clinical conventional urine culture. The sensitivity and specificity of the real-time PCR in these clinical samples were 92% and 87%, respectively. The test results were quantitative, and allowed distinguishing between significant bacteriuria (i.e. 10^5 cfu/ml) and low-count bacteriuria that might have been due to contamination.
To test a study urine sample, one ml of urine was centrifuged at 16,250 x g for five minutes. The pellet was washed twice, suspended in one ml of sterile injection water, and 100 μL of the suspension was heated for two minutes at 1,000 Watt in a microwave oven for DNA-preparation. Five microliter (μL) was added to the real-time PCR reaction volume as DNA template. Each 25 μL reaction volume consisted of 12.5 μL 2x Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) that contains AmpliTaq Gold DNA polymerase, 300 nM forward primer (5'-ACCGACATCGTGGTGATGC-3'), 300 nM reverse primer (5'-AGCAACAGTTCAGCAAAGTCCA-3'), and 175 nM probe (5'-CATTATGTGTCGTCGCGGCTTCGAA-3'); the DNA template was the last ingredient added. The ABI PRISM 7700 Sequence Detection System (PE Biosystems, Nieuwerkerk aan de IJssel, the Netherlands) was used for the real-time PCRs.

Cycling parameters were first the uracil-*N*-glycosylase (UNG) reaction at 50°C for two minutes, then AmpliTaq Gold activation at 95°C for ten minutes, followed by 45 cycles of denaturation at 95°C for fifteen seconds - combined annealing and extension at 60°C for one minute. Emitted fluorescence from each well was measured during both the denaturation and annealing/extension steps in every cycle. Amplification plots were constructed using the ABI PRISM 7700 Sequence Detection System software, version 1.7 (PE Biosystems).

Renal function

We used the Cockroft-Gault formula to estimate the creatinine clearance (in ml per minute) as a proxy for the glomerular filtration rate (GFR).[1] In addition, the calculated GFR was divided in five stages (according to the National Kidney Foundation classification): at least 90 ml per minute (stage 1), 60 to 89 ml per minute (stage 2), 30 to 59 ml per minute (stage 3), 15 to 29 ml per minute (stage 4), or less than 15 ml per minute (stage 5).[1]

Data analysis

The present results are based on a cohort study of 490 women who were followed for renal function development, and a nested case-control study to study end-stage renal failure, both in relation to *E. coli* bacteriuria at baseline.
Baseline characteristics are compared between women with and without bacteriuria. Comparisons between means were performed with the Student's t test, and comparisons between nominal or categorical data with the Chi square test. Linear regression analysis was used to calculate the difference in creatinine clearance between women with versus women without bacteriuria. The distribution over five stages of renal function (based on the GFR) according to women with and without bacteriuria was tested with the Mann-Whitney U test. In the case-control analysis, the relative risk of renal failure in the presence versus absence of bacteriuria was estimated by the odds ratio (OR) and 95% confidence intervals (CI) as calculated using logistic regression. Adjustments were made for potential confounding factors, i.e. age and weight.

Results

Cohort analysis

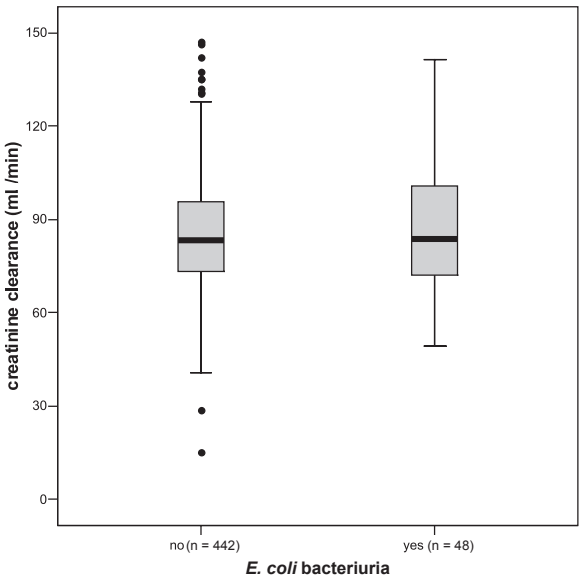
The baseline characteristics of the follow-up cohort are given in *table 1*. Forty-eight of 490 women (10%) were classified with *E. coli* bacteriuria at baseline. No correlation was found between bacteriuria and age (*Table 1*). At baseline, the use of antibiotics was the only factor that significantly differed between women with and without bacteriuria (33 versus 11 %, $p = 0.047$).
At study endpoint, the mean creatinine clearance for women with baseline bacteriuria and without baseline bacteriuria was 87 ± 21 and 85 ± 18 ml per minute, respectively (*Figure 2* and *Table 2*). In the multivariate analysis, *E. coli* bacteriuria at

Table 1 Cohort study: Baseline characteristics of women with (n = 48) and women without (n = 442) *E. coli* bacteriuria[1]

	E. coli bacteriuria		p value
	absent (n = 442)	present (n = 48)	
Age (year)	45.0 ± 3.2	45.4 ± 3.2	.35
Postmenopausal	102 (23%)	12 (25%)	.77
Body Mass Index (BMI) (kg/m²)	24.2 ± 3.5	24.9 ± 3.6	.21
Married or living with partner (n = 377)	303 (90%)	37 (95%)	.30
Given birth to living child(ren)	392 (89%)	45 (94%)	.28
Antibiotics (n = 113)	11 (11%)	3 (33%)	.047

Values are given as mean ± standard deviation, or as number of patients (and percentage),[1] the total number of study subjects is 490 unless otherwise stated.

Figure 2



baseline was not associated with creatinine levels at follow-up, adjusted for age and weight (p = 0.71). Finally, the distribution in stages of renal function was not different for women with bacteriuria compared to women without bacteriuria (*Table 2*; p = 0.68).

Case-control analysis

The baseline characteristics of the women who developed renal failure and the control subjects, including the results of the real-time PCR of their urine, are

Table 2 Cohort study: Follow-up results on renal function of women with and without *E. coli* bacteriuria at baseline (univariate analysis)

	E. coli bacteriuria		p value
	absent (n = 442)	present (n = 48)	
Creatinine (µmol/l)	73.2 ± 18.4	72.5 ± 9.8	.68
Creatinine clearance (ml/min)	85 ± 18	87 ± 21	.69
Stage of renal function			.55
creatinine clearance ≥ 90 ml/min	153 (35%)	19 (40%)	
creatinine clearance 60–89 ml/min	273 (62%)	26 (54%)	
creatinine clearance 30–59 ml/min	14 (3%)	3 (6%)	
creatinine clearance 15–29 ml/min	2 (<1%)	0	
creatinine clearance < 15 ml/min	0	0	

Values are given as mean ± standard deviation, or as number of patients (and percentage).

presented in *Table 3*. Compared to the women in the control group, women who developed renal failure were younger at baseline (mean age 52 versus 56 years), were more likely to be premenopausal (37% versus 22%) and used antihypertensive medication more often (49% versus 9%; all comparisons $p < 0.05$). No difference in duration until kidney replacing therapy was found between bacteriuric and nonbacteriuric individuals (14.6 versus 13.7 years, $p = 0.80$).
Seven of 49 women who developed renal failure had *E. coli* bacteriuria at baseline, compared to 29 of 206 women in the control group (both 14%). The odds ratio for the development of renal failure in the presence of *E. coli* bacteriuria, corrected for age, was 1.1 (95% CI 0.4–2.8, $p = 0.86$).

Table 3 Case-control study: Baseline characteristics of women who developed renal failure (cases, n = 49) and the controls (n = 206)

	cases	n	controls	n	p value
Age (year)	51.7 ± 6.6	49	55.7 ± 4.2	206	< .01
Body Mass Index (BMI) (kg/m²)	26.9 ± 5.7	49	26.0 ± 5.7	206	.14
Married / living with partner	42 (93%)	45	182 (88%)	206	.33
≥1 life born child(ren)	41 (84%)	49	158 (77%)	206	.29
Postmenopausal	31 (63%)	49	160 (78%)	206	.04
Hormone replacing therapy	4 (9%)	45	26 (13%)	206	.48
Oral contraceptives	1 (2%)	45	1 (1%)	173	.30
Anti diabetes medication or diet	3 (10%)	30	12 (6%)	206	.38
Antihypertensive medication	24 (49%)	49	19 (9%)	206	< .01
***E. coli* bacteriuria**	7 (14%)	49	29 (14%)	206	.97

Values are given as mean ± standard deviation, or as number (and percentage).

Discussion

In this prospective cohort study in a population of healthy adult women, no association was found between *E. coli* bacteriuria and renal function deterioration or the development of end-stage renal failure after a mean follow-up of 12 and 14 years, respectively.

Among the strengths of our study are its size and the length of follow-up. Almost 500 women were evaluated for the development of chronic kidney during a mean follow-up of 12 years. The baseline cohort consisted of over 38 thousand potential study subjects, which allowed us to identify approximately 50 women who developed end-stage renal failure. The control group originated from the same study population minimizing selection bias, and the national registry of RENINE enabled us to be sure that none of these controls received kidney replacing therapy.

The limitations include that we had to rely on only one urine sample to define bacteriuria. However we, and others, have validated this before.[17] We made the assumption that however bacteriuria might be transient in a proportion of the bacteriuric study subjects, bacteriuria at one point reflects a higher susceptibility to recurrent and persistent bacteriuria in general, even after antimicrobial therapy. Previous findings are supportive with this assumption. A Swedish study among 1462 adult women showed that women with bacteriuria at study entry had an increased risk of having bacteriuria six and twelve years later, compared to women without bacteriuria (OR 6.9 and 3.1, after six and twelve years, respectively, both $p < 0.01$).[18] Others showed a strong correlation between a history of UTIs and a current UTI among sexually active women.[4] Another limitation of our methods is that some of the urine samples may have become contaminated before storage, however this most likely has effected cases and controls in the same proportion. Moreover, contamination usually leads to the growth of more pathogens, often non-*E. coli*, with lower colony counts, which are not picket up by our real-time PCR. Pyuria could no more be determined in urine specimen stored since the 1970's.

Although the urinary tract is normally sterile, bacteriuria is very common, especially in the female and the elderly population. Previous studies reported a prevalence of asymptomatic bacteriuria of 5% in sexually active young women and over 20% in the ambulatory elderly. *E. coli* is the most common infecting uropathogen.[4,5,18] The present study similarly showed prevalences of *E. coli* bacteriuria of 10% and 14%.

Results from previous *in vitro* and *in vivo* studies indicate that a UTI with *E. coli* can lead to renal damage, either by the microorganism itself or by the following host response. For instance, it has been shown that type 1 fimbriae (the adhesive organelles at the outer surface of the bacterial membrane) can cause scarring in the renal parenchyma of rats, with large foci of inflammation.[7] This might be due to the activation of polymorphonuclear leukocytes by type 1 fimbriated-strains, which leads to the release of tissue destroying enzymes.[19] Mice models have shown that although neutrophils are important in bacterial clearance, they can also cause renal damage.[20] In a recent follow-up study, renal scarring was detected in 29 of

63 adult women ten to twenty years after hospitalization for pyelonephritis.[21] In contrast, no study has convincingly shown that (asymptomatic) bacteriuria can lead to a clinically relevant decline in renal function in otherwise healthy women.[18,22] Our longitudinal findings in a large cohort of women give strong support to the absence of such association. As an explanation, Svanborg et al. found that certain *E. coli* strains stop expressing adherence factors like type 1 and P fimbriae once they have established bacteriuria. Therefore, these strains can remain present in the bladder without triggering an inflammatory response from the host and without causing damage.[20]

We determined the presence of *E. coli* bacteriuria and cannot exclude that the presence of other uropathogens in urine can lead to renal scarring. For instance, *Klebsiella* species possess type 1 fimbriae that are antigenically related to the fimbriae found in *E. coli*.[23] However, *Klebsiella* is the causative organism in only a small minority of urinary tract infections.

Numerous studies have evaluated factors predisposing to asymptomatic bacteriuria and symptomatic UTIs, such as female gender, higher age, and diabetes.[4,5,24] In accordance with Hooton et al.[4], we found no association between age and bacteriuria at baseline, possibly due to the small range in age. In the baseline cohort, the presence of diabetes was only established in a minority of participants. Unfortunately, for both the cohort study and the case-control study the small number of diabetic patients made it impossible to draw conclusions on the possible influence of bacteriuria on renal function in this subpopulation. We did find a higher use of antibiotics among the women with bacteriuria compared to women without bacteriuria. This is suggestive of a symptomatic UTI in these women at time of inclusion, but data on symptoms of a UTI are lacking.

In conclusion, the findings ofz our long-term follow-up study among a large cohort of healthy adult women do not support the view that *E. coli* bacteriuria leads to loss of renal function or the development of end-stage renal failure.

Acknowledgements

We thank "Stichting RENINE" for giving us the opportunity to use their data.
This project has been supported by the Foundation "De Drie Lichten", the Netherlands.

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Nine

Escherichia coli bacteriuria in female adults is associated with the development of hypertension

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Submitted for publication

Abstract

Introduction

To investigate whether *Escherichia coli* bacteriuria is associated with the development of hypertension during a long-term follow-up.

Methods

A prospective cohort study was performed among the participants of two population based studies. Between 1974 and 1986 all women, aged 39 to 68 years old, who lived in the city of Utrecht, the Netherlands, were invited to participate in a breast-cancer-screening program. The participants completed a questionnaire, underwent a medical examination, and collected a midstream morning urine sample that remained stored since. From 1993 to 1997 another population-based study was performed in the same area. We performed a full cohort analysis for 444 women who participated in both studies. *E. coli* bacteriuria was diagnosed by a real-time Polymerase Chain Reaction. Hypertension was defined as the (previous) use of antihypertensive medication, and/or a measured systolic blood pressure of at least 160 mmHg or a diastolic blood pressure of 95 mmHg or higher. The mean follow-up was 11.5 ± 1.7 years.

Results

Forty women (9%) had *E. coli* bacteriuria at baseline. Women who had *E. coli* bacteriuria at baseline had a mean blood pressure at study endpoint of 133 ± 20 mmHg systolic and 78 ± 11 mmHg diastolic, and women without bacteriuria had values of 129 ± 20 and 78 ± 11 mmHg, respectively (p value for difference 0.33 and 0.88). Although *E. coli* bacteriuria was not associated with the blood pressure as a continuous variable, it was associated with the development of hypertension during follow-up (odds ratio 2.8, 95% confidence interval 1.4–5.5). This association remained statistically significant after correction for age, weight and creatinine.

Conclusion

E. coli bacteriuria may increase the risk of future hypertension.

Introduction

Although the urinary tract is normally sterile, (asymptomatic) bacteriuria is a common phenomenon, especially in women. Different studies report a prevalence of asymptomatic bacteriuria of approximately 5% among healthy young women, increasing to over 20% in the elderly.[1,2] *Escherichia coli* is the most prevalent uropathogen.[3] Several authors in the first half of the twentieth century have suggested a role of bacteriuria in the etiology of hypertension.[4] Although more recent studies also found a correlation, no prospective study has convincingly shown that bacteriuria on itself leads to hypertension.[5]
The present study aims to address the question whether *E. coli* bacteriuria is associated with an increased risk of the development of hypertension during a long-term follow-up period. In addition, the risk of a heart attack or stroke in the presence of bacteriuria is studied.

Methods

Study population

A full cohort analysis was performed for women who participated in two population based studies. Between 1974 and 1986 all women, born between 1911 and 1945, who lived in the city of Utrecht and surroundings, the Netherlands, were invited for a breast-cancer-screening program, with a participation rate of 68 to 72%.[6] A total number of 38,994 women, aged 39 to 68 years old at intake, participated (the baseline cohort). Baseline measurements, performed between 1974 and 1986, included extensive questionnaires, a short medical examination, and the collection of a midstream morning urine sample. Data obtained through the questionnaires included age, marital status, parity, menopausal age, diet and drug use. During the medical examination weight, height and the blood pressure were measured. Approximately 200 ml urine was stored in plastic polypropylene jars, without preserving agents, and stored at –20°C for future analyses.[7] All women gave oral consent to use their data and urine samples for future scientific research.
From 1993 to 1997, participants in the breast-cancer-screening program received an invitation by mail to join an additional study to assess the relation between nutrition and cancer and other chronic diseases, the Prospect-EPIC study (the follow-up cohort). A total of 17,357 women living in Utrecht and surroundings agreed to take part (participation rate 34.5%).[8] Participants were between 49 and 70 years old at enrolment. Information was collected on the basis of two self-administered questionnaires and a medical examination including blood pressure. Non-fasting blood samples were successfully drawn from 97.5% of the women, and stored under liquid nitrogen at –196°C. Later, from these samples the serum creatinine level was measured. Approximately 88% of the women signed a detailed informed consent, enabling the researchers to use their blood samples for future analysis, and to obtain information on future morbidity and mortality.[8]

In total, 506 women who participated in both the baseline cohort and the follow-up cohort were included. Sixty-two women had to be excluded for the following reasons: a missing urine sample (n = 13), the use of antihypertensive medication at baseline (n = 45), kidney transplantation during follow-up (n = 1), or missing data on hypertension at study endpoint (n = 3). Finally, 444 women were included in a prospective study to assess the relation between bacteriuria and the development of hypertension. The mean duration of follow-up was 11.5 ± 1.7 years, ranging from 8.3 to 18.6 years from baseline until participation in the follow-up study (and was not different for women with compared to women without bacteriuria).
This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands.

E. coli bacteriuria

E. coli bacteriuria was defined as the presence of 10^5 colony forming units (cfu) of *E. coli* per milliliter (ml) of urine. It was diagnosed by a real-time Polymerase Chain Reaction (PCR) that we developed and validated beforehand, a technique that was also used by others.[9,10] Briefly, PCR primers and probe complementary to regions of the gadA gene specific for *E. coli* were designed for the real-time PCR assay. The laboratory sensitivity and specificity of the real-time PCR tested with 50 *E. coli* strains and with 41 non-*E. coli* strains (including the most prevalent uropathogens and many members of the vaginal and anal flora) were 100% (50/50) and 98% (40/41), respectively. For clinical evaluation, 42 clinical urine specimens (12 with and 30 without *E. coli*) were tested and the results were compared to those of a clinical conventional urine culture. The sensitivity and specificity of the real-time PCR in these clinical samples were 92% and 87%, respectively. The test results were quantitative, and allowed distinguishing between significant bacteriuria (i.e. 10^5 cfu/ml) and low-count bacteriuria that might have been due to contamination.
To test a study urine sample, one ml of urine was centrifuged at 16,250 x g for five minutes. The pellet was washed twice, suspended in one ml of sterile injection water, and 100 μL of the suspension was heated for two minutes at 1,000 Watt in a microwave oven for DNA-preparation. Five microliter (μL) was added to the real-time PCR reaction volume as DNA template. Each 25 μL reaction volume consisted of 12.5 μL 2x Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) that contains AmpliTaq Gold DNA polymerase, 300 nM forward primer (5'-ACCGACATCGTGGTGATGC-3'), 300 nM reverse primer (5'-AGCAACAGTTCAGCAAAGTCCA-3'), and 175 nM probe (5'-CATTATGTGTCGTCGCGGCTTCGAA-3'); the DNA template was the last ingredient added. The ABI PRISM 7700 Sequence Detection System (PE Biosystems, Nieuwerkerk aan de IJssel, the Netherlands) was used for the real-time PCRs. Cycling parameters were first the uracil-*N*-glycosylase (UNG) reaction at 50°C for two minutes, then AmpliTaq Gold activation at 95°C for ten minutes, followed by 45 cycles of denaturation at 95°C for fifteen seconds - combined annealing and extension at 60°C for one minute. Emitted fluorescence from each well was

measured during both the denaturation and annealing/extension steps in every cycle. Amplification plots were constructed using the ABI PRISM 7700 Sequence Detection System software, version 1.7 (PE Biosystems).

Blood pressure, heart attack, stroke

Blood pressure was measured at follow-up with a standard mercury sphygmomanometer after the subject had been seated for five minutes. Hypertension was defined as the (previous) use of antihypertensive medication (assessed at follow-up by the question: "have you ever been treated with drugs for high blood pressure?"), and/or a measured systolic blood pressure of at least 160 mmHg or a diastolic blood pressure of 95 mmHg or higher. A history of having had a heart attack or stroke was assessed at follow-up by the two additional questions: "Have you ever had a heart attack / stroke?".

Data analysis

The present results are based on a cohort study of 444 women who were followed for the development of hypertension in relation to *E. coli* bacteriuria at baseline.
Baseline characteristics are compared between women with and without bacteriuria. Comparisons between means were performed with the Student's t test, and comparisons between nominal or categorical data with the Chi square test. Linear regression analysis was used to calculate the adjusted difference in blood pressure between women with versus women without bacteriuria. The relative risk of hypertension in the presence of bacteriuria was estimated by logistic regression and quantified as odds ratios (OR) and 95% confidence intervals (CI). Adjustment was done for potential confounding factors, i.e. age, weight and serum creatinine level.

Results

Baseline characteristics of the total study group (444 women) are presented in *table 1*, as well as the comparisons between women with (n = 40, 9%) and without (n = 404, 91%) *E. coli* bacteriuria at baseline. The use of antibiotics was the only factor that significantly differed between women with and without bacteriuria (10% versus 33%, p = 0.04). Eight of the 45 women (18%) who had to be excluded because of the use of antihypertensive medication at baseline had *E. coli* bacteriuria, which was more than the percentage of 9% of the final study group without antihypertensive drugs at baseline (p = 0.06).
At measurement after a mean follow-up of 11.5 years, women who had *E. coli* bacteriuria at baseline had a mean blood pressure at study endpoint of 133 ± 20 mmHg systolic and 78 ± 11 mmHg diastolic, and women without bacteriuria had values of 129 ± 20 and 78 ± 11 mmHg, respectively (p-value for difference 0.33 and 0.88). Although *E. coli* bacteriuria was not associated with the blood pressure as a continuous variable, it was associated with the development of hypertension (OR

2.8, 95% CI 1.4–5.5) (*Table 2*). This was mainly due to more bacteriuric women that started antihypertensive drugs when compared to nonbacteriuric participants. The association remained statistically significant after correction for age, weight and creatinine (OR 2.8, 95% CI 1.4–5.6).
Within a subgroup analysis with the group of 108 women of who was known whether they used antibiotics at baseline the same trend was visible, also after correction for antibiotic use, although it lost statistical significance (data not shown). *E. coli* bacteriuria was not associated with the different classes of anti-hypertensive drugs (data not shown). The incidence of heart attacks or strokes was not increased among women with bacteriuria at baseline (*Table 2*).

Table 1 Baseline characteristics of the total cohort study, and separately for women with versus without *E. coli* bacteriuria

	total study group (n = 447) [1]	**no *E. coli* bacteriuria (n = 404)**	***E. coli* bacteriuria (n = 40)**	**p value**
Age (year)	44.9 ± 3.2	44.9 ± 3.2	45.3 ± 3.4	.51
Body Mass Index (BMI) (kg/m²)	24.0 ± 3.2	23.9 ± 3.2	24.6 ± 3.6	.26
Postmenopausal	98 (22%)	88 (22%)	10 (25%)	.64
Married or living with Partner (n = 336)	303 (90%)	273 (90%)	30 (97%)	.20
Given birth to living child(ren)	401 (90%)	363 (90%)	38 (95%)	.29
Antibiotics (n = 108)	13 (12%)	10 (10%)	3 (33%)	.04

Values are given as mean ± standard deviation, or as number of patients (and percentage), [1] the total number of study subjects is 447 unless otherwise stated.

Table 2 Follow-up characteristics of the cohort study divided into women with and without *E. coli* bacteriuria at baseline (n = 444 unless otherwise stated)

	no *E. coli* bacteriuria (n = 404)	***E. coli* bacteriuria (n = 40)**	**odds ratio (95% CI)[1]**	**p value**	**adjusted p value[2]**
Hypertension[3]	78 (19%)	16 (40%)	2.8 (1.4–5.5)	.003	.003
Antihypertensive medication	49 (12%)	10 (25%)	2.4 (1.1–5.2)	.03	.03
Systolic RR ≥ 160 and/or diastolic RR ≥ 95 mmHg	45 (11%)	9 (23%)	2.3 (1.0–5.2)	.04	.04
Systolic RR > 140 and/or diastolic RR > 90 mmHg	112 (28%)	14 (35%)	1.4 (0.7–2.8)	.33	.40
Heart attack ever (n = 443)	4 (1%)	1 (3%)	2.6 (0.3–23.5)	.41	.59
Stroke ever	7 (2%)	1 (3%)	1.5 (0.2–12.1)	.73	.72

Values are given as mean ± standard deviation, or as number of patients (and percentage); [1] CI indicates confidence interval, [2] Adjustments were made for age, weight and serum creatinine, [3] for definition see Methods.

Discussion

In this prospective cohort study in a population of healthy adult women, we found a correlation between *E. coli* bacteriuria and the prevalence of hypertension 12 years later.

Among the strengths of our study are its size and the length of follow-up. The breast-cancer-screening program which formed the baseline cohort consisted of over 38 thousand potential study subjects of whom a urine sample was stored, which gave us the unique opportunity to study bacteriuria and its long-term consequences.

The limitations include the fact that we had to rely on only one urine sample to define bacteriuria. However we, and others, have validated this before.[11] We made the assumption that however bacteriuria might be transient in a proportion of the bacteriuric study subjects, bacteriuria at one point reflects a higher susceptibility to recurrent and persistent bacteriuria in general, even after antimicrobial therapy. Previous findings are supportive with this assumption.[1,12] Some of the urine samples may have become contaminated before storage, however this will be equally divided among the total study group. Moreover, contamination usually leads to the growth of more pathogens, often non-*E. coli*, with lower colony counts, which are not picked up by our real-time PCR. *E. coli* is the causative microorganism found most prevalent, and the prevalence of *E.coli* bacteriuria of 9% among middle-aged women reported here is in the range of what could be expected beforehand. The baseline data included the use of any drugs which allowed us to exclude women who used antihypertensive medication. However, blood pressure was not measured at baseline, and therefore the study cohort will include some women with undiagnosed hypertension. At follow-up we had to rely on a single blood pressure measurement. But we assume that the incidence of increased blood pressure due to other reasons will be equally divided among women with and without bacteriuria at baseline. Moreover, the increased prevalence of hypertension in the group of bacteriuria was mainly due to more women that started antihypertensive drugs in this group compared to the group of women without bacteriuria.

Several authors in the first half of the twentieth century have suggested a role of bacteriuria in the etiology of hypertension, as reviewed before.[4] For instance, Kass showed small differences in blood pressure between bacteriuric and nonbacteriuric women aged 15 to 64 years old.[13] Although more recent studies also found a correlation, no prospective study has convincingly shown that bacteriuria in itself leads to hypertension.[5] In our cohort study, we found a higher prevalence of hypertension in the bacteriuric group after 12 years of follow-up. The underlying mechanism of this finding is not clear. Hypertension is a lasting increase in blood pressure with a heterogeneous etiology consisting of both genetic and environmental factors. Patients share the inability to excrete sodium at a normal arterial pressure.[14] If bacteriuria would lead to hypertension, the most attractive explanation would be that hypertension arises secondary to renal scarring caused by the (type 1 fimbriae of the) uropathogens. In the multivariate analysis, correction for creatinine did not change the results, but hypertension can occur before the

reduction in creatinine clearance becomes apparent (for example in chronic glomerulonephritis).[15] The absence of a change in odds ratio after adjustment for age, weight and creatinine confirms the notion that these factors were not related to bacteriuria. An alternative explanation is that both bacteriuria and hypertension are found more frequently among individuals with comorbidity or that they share a same (currently unknown) cause. This is supported by the higher prevalence of bacteriuria among women who used antihypertensive drugs at baseline.
In conclusion, in this prospective study a strong correlation was found between *E. coli* bacteriuria and hypertension after 12 years follow-up. Given the importance of hypertension the nature of this correlation needs to be studied in future studies.

Acknowledgement

This project has been supported by the Foundation "De Drie Lichten", the Netherlands.

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Ten

Treatment of asymptomatic bacteriuria in diabetic women

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N Engl J Med 2003;348(10):957-8

Harding et al. concluded that treatment of asymptomatic bacteriuria (ASB) in women with diabetes does not appear to reduce complications and that screening and treatment are therefore not needed.[1] However, the results of their study show that the women in the placebo group received significantly more antibiotics for symptomatic urinary tract infections (UTIs) than women in the antimicrobial-therapy group. In addition, in our opinion, it is the effect of ASB on renal function, and not the development of symptomatic UTI, that is the most important variable. In citing our study,[2] Harding et al. do not mention that we reported that women with diabetes mellitus (DM) type 1 and ASB had a tendency toward a decline in renal function over a short follow-up period. Since DM type 1 and type 2 are considered different diseases, separate analyses are warranted. In the study by Harding et al., all patients were analyzed together and only 21 patients (20%) had DM type 1.

The conclusion of this interesting study should be that it is difficult to keep these patients free of bacteriuria. Furthermore, we believe it is premature to conclude that screening and treatment of ASB in diabetic women are not needed. We are awaiting the results of our five-year follow-up study of nearly 200 women with DM type 1, in which renal function decline is the primary outcome variable.

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Eleven

Asymptomatic bacteriuria in women with diabetes mellitus: effect on renal function after 6 years follow-up

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Submitted for publication

Abstract

Introduction

Women with diabetes mellitus (DM) have an increased prevalence of asymptomatic bacteriuria (ASB). Previously, we reported that women with DM type 1 and ASB showed a tendency to a faster decline in renal function than women without ASB after 18 months, but the long-term consequences of ASB on renal function in diabetic women are unknown.

Methods

A prospective study was performed among women with DM type 1 or type 2, aged 18 to 75 years. Women with ASB at baseline were compared to women without ASB for differences in renal function development and the incidence of hypertension. The Cockcroft-Gault equation was used to estimate the creatinine clearance at baseline and study endpoint.

Results

A total of 644 women were included, 296 women with DM type 1 and 348 women with DM type 2, with a mean age of 51 ± 15 years. The mean duration of follow-up was 6.1 ± 1.9, ranging from 1.0 to 8.3 years. At baseline, the prevalence of ASB was 17%. *E. coli* was cultured in 67% of the positive urine samples. In women with DM and ASB, the creatinine clearance decreased from 87 ml/min at baseline to 76 ml/min at study endpoint; in diabetic women without ASB the creatinine clearance decreased from 97 to 88 ml/min. In the univariate analysis, ASB was associated with a higher relative decrease in creatinine clearance (14 ± 22% and 9 ± 23% in women with versus women without ASB, respectively, $p = 0.03$), but not with the absolute decrease in creatinine clearance (12 ± 19 and 9 ± 20 ml/min, respectively, $p = 0.12$). However, in the multivariate analyses, according to age strata, and including the length of follow-up, duration of DM, and microalbuminuria at baseline, no association was found between ASB and the relative or the absolute decrease in creatinine clearance. Also when women with DM type 1 and women with DM type 2 were analyzed separately, no association was found between ASB and renal function. Women with ASB developed hypertension more often than women without ASB (54 versus 37%, $p = 0.045$), but in the multivariate analysis, including age, duration of DM, and length of follow-up, the association between ASB and hypertension disappeared ($p > 0.2$).

Conclusion

Women with DM (type 1 or type 2) with ASB do not have an increased risk for a faster decline in renal function or the development of hypertension after 6 years of follow-up.

Introduction

Chronic kidney disease is an increasing public health problem.[1] The prevalence in the United States is estimated to be approximately 11% of the adult population.[2] Diabetes Mellitus (DM) is one of the main causes of kidney disease and end-stage renal failure.[1] In the Netherlands, DM is registered as the primary diagnosis in 16% of all new cases of renal replacement therapy, whereas in the United States it is the primary diagnosis in 44% of the cases.[3,4] Vascular complications are the most common cause of diabetic nephropathy, but it is possible that urinary tract infections (UTIs) also contribute to renal insufficiency in diabetic patients.
Women with DM have an increased prevalence of asymptomatic bacteriuria (ASB), but also an increased risk of symptomatic UTIs and developing complications of UTIs such as renal abscesses.[5-7] It was also shown that at short term follow-up treatment of ASB in women with DM did not appear to reduce complications.[8] *Escherichia coli* is the leading uropathogen in nondiabetic as well as in diabetic patients. Ninety percent of *E. coli* possesses type 1 fimbriae, the adhesive organelles found at the outer bacterial membrane. We have shown *in vitro* that type 1-fimbriated *E. coli* have an increased adherence to uroepithelial cells voided by women with DM (*see chapter 3*).[9] Others demonstrated that UTIs with type 1-fimbriated *E. coli* can lead to scar formation in the renal parenchyma of infected rats.[10] At present, conclusive and prospective data with a long follow-up period directly relating ASB (with *E. coli*) to long-term risk of renal failure in diabetic patients are lacking. In a previous study, we showed that women with DM type 1 and ASB showed a tendency toward a faster decline in renal function than women without ASB after 18 months follow-up.[11] Taken together, we hypothesized that ASB in women with DM could lead to a faster decline in renal function, and decided to enlarge our cohort of diabetic women and to prolong the follow-up period. Besides the effects on renal function, we also studied the influence of ASB on the development of hypertension. The results are presented here.

Research design and methods

Patients

Patients were enrolled during two periods, between October 1996 and November 1997, and between August 2000 and June 2002. All participants visited the diabetes outpatient clinics of the University Medical Center Utrecht (tertiary care hospital), three non-university hospitals (Jeroen Bosch Hospital 's Hertogenbosch, Diakonessenhuis Utrecht, Catharina Hospital Eindhoven) or the offices of five general practitioners. Patients were asked to participate by their treating physician and enrolled by one of the investigators. Women aged 18 to 75 years were eligible to participate if they had either DM type 1 or DM type 2 (defined by the treating physician). Exclusion criteria were: pregnancy, recent hospitalization or surgery (< four months), known urinary tract abnormalities, symptoms of a UTI, or the use of antimicrobial drugs in the previous fourteen days. The study had the approval of

the Medical Ethical Committee of all hospitals. All patients gave written informed consent.

Data collection

All patients were interviewed at baseline using a standardized questionnaire including age, number of UTIs in the previous year, pregnancies, urinary tract surgery, recent sexual intercourse (< one week), contraceptive use, menopausal status, and use of (local) estrogens, as published before.[5] Their medical records were reviewed at baseline and at study closure to collect additional information on type and duration of DM, secondary complications of DM (retinopathy, neuropathy, macrovascular complications), and medication. Blood pressure, weight, and height were recorded at baseline and study closure. Serum creatinine, hemoglobin A1c (HbA1c), and urinary albumin excretion measurements were extracted from the electronic patient database. The mean time from the date of creatinine measurement until inclusion was 44 days (SD ± 163 days). Additionally, the last creatinine measurement before the end of study, i.e. January 1st 2005, was noted, and the date of blood withdrawal was taken as study endpoint for that individual. In case a patient died or needed kidney replacing therapy, the last ambulatory creatinine value was noted. The Cockcroft-Gault equation was used to estimate the glomerular filtration rate (GFR).[2]

Urine specimens

All women who were included in the first inclusion period (1996–1997) were asked to provide two midstream urine specimens, one at baseline, and one in the following four months. The women who were included in the second period (2000–2002) were asked to provide up to six urine specimens: the first at baseline, the second after one week, and the next four simultaneously with the routine visits to the treating physician (with a time interval of approximately three to four months). For the second culture, the women included in the second period were provided with a uricult (Orion Diagnostica, Espoo, Finland) and a return envelope. For all other cultures fresh voided specimens were collected. Finally a sample of the total study group was asked to provide an additional midstream urine sample at study endpoint.

Quantitative urine culturing was performed as described before.[11] Causative microorganisms were identified using Vitek automated identification System (bioMérieux, Den Bosch, the Netherlands). When growth of three or more different microorganisms was seen, the urine specimen was regarded as being contaminated. All patients and their physicians were blinded for the culture results.

Leukocyturia was determined directly from an uncentrifuged midstream urine sample by microscopy (400 x magnification), or by a leukocyte esterase test (Combur-Test; Boehringer Mannheim, Almere, the Netherlands).

Definitions

ASB was defined as the presence of at least 10^5 cfu/ml of one or two known uropathogen(s) in a urine culture from a patient without symptoms of a UTI or fever.

Albumin excretion was measured in a 24-h urine sample, or the albumin-creatinine ratio in a single-void urine specimen was calculated. Normoalbuminuria was defined as an albumin excretion of less than 20 mg/l or an albumin-to-creatinine ratio < 2.5 g/mol, microalbuminuria as an albumin excretion of 20 to 200 mg/l or an albumin-to-creatinine ratio of 2.5 to 30 g/mol, and macroalbuminuria was defined as an albumin excretion of more than 200 mg/l or an albumin-to-creatinine ratio higher than 30 g/mol.

The relative increase in creatinine and creatinine clearance was defined as the difference between the values at study endpoint and the baseline values, divided by the baseline values and multiplied by 100. In addition, the absolute differences in creatinine level and creatinine clearance between baseline and endpoint were calculated.

Peripheral neuropathy was defined as at least one positive test result of a standardized vibration, temperature, or monofilament test, or (when these tests were not performed) the presence of at least four of the following symptoms: complaints of pain, burning, pricking, numbness, or tingling sensations in the feet, an absence of ankle jerks, disturbances in pinprick or light touch sense, or feet abnormalities (deformation, callus, ulcer, fissure).

Blood pressure was measured with a standard mercury sphygmomanometer after the subject had been seated for five minutes. Hypertension was defined as a systolic blood pressure higher than 140 mmHg, and/or a diastolic blood pressure higher than 90 mmHg, and/or the use of anti-hypertensive drugs.

Statistical analysis

Absolute and relative values between baseline and follow-up were compared between patients with and those without ASB, using the t test for continuous, the Mann-Whitney U test for categorical, and the Chi square test for dichotomous variables. Because the Cockcroft-Gault formula for the estimation of the creatinine clearance includes age, adjusting for age in a multivariate model is not possible. Therefore patients were stratified into three age strata to assess the impact of age on the association between ASB and the (relative increase in the) creatinine clearance (respectively 18 to 36, 37 to 55, and 56 to 75 years old). All analyses were performed on the entire study population and on women with DM type 1 and DM type 2 separately. Linear and logistic regressions were used respectively to calculate the differences in blood pressure and the relative risk of hypertension in the presence or absence of bacteriuria. Women with hypertension at baseline were excluded from the latter analyses. A p value of < 0.05 was considered statistically significant.

Results

Baseline characteristics

A total of 716 diabetic women were enrolled in this study, of which 72 women had to be excluded for the following reasons: no uncontaminated urine specimen (n = 8), no creatinine measurement at baseline or at study endpoint (n = 27), an anatomic kidney abnormality (n = 8), or withdrawal from the study within twelve months (n = 29). The final study cohort consisted of 644 women, 296 women with DM type 1 and 348 women with DM type 2. The mean duration of follow-up was 5.8 ± 2.1 (median 7.0) and 6.4 ± 1.8 (median 7.1) years for women with DM type 1 en DM type 2, respectively (ranging from 1.0 to 8.3 years).
Baseline characteristics of all women together, those with DM type 1, and those with DM type 2, are given in *Table 1*. Women with DM type 1 were younger, but had a longer duration of DM, than women with DM type 2. At baseline, 201 women with DM type 2 (58%) were treated with insulin only, 97 (28%) with oral hypoglycemic medication only, 41 (12%) with a combination of both, and five women (2%) were on a diet only (data were incomplete for four women).

Asymptomatic bacteriuria

Two (or more) cultures were available from 516 of the 644 women. Of these women, 443 women had either two positive cultures with the same microorganism (n = 47, 11%), or two negative cultures (n = 396, 89%). A total of 73 women had two urine cultures with different results: either one positive or one negative culture (n = 68), or two positive cultures with different microorganisms (n = 5). Of the remaining 128 women of whom only one culture was available, 23 women had a positive culture (18%), whereas 105 had a negative culture result (82%). There were no differences in clinical characteristics between the women with two positive cultures and the women who had a positive first culture, but a second culture that was either negative, not available or positive with another uropathogen (for all comparisons p > 0.1). Therefore, we decided to base the presence of ASB on the results of the first collected culture, in other words when the first collected urine culture was positive the woman was defined as having ASB.
At baseline, the prevalence of ASB was 17% for the total study group. The prevalence was lower in women with DM type 1 when compared to women with DM type 2 (12% and 21%, respectively), but multivariate analysis revealed that this was due to the difference in age. *E. coli* was the causative uropathogen in 74 of the 110 bacteriuric women (67%). Other isolated microorganisms included: enterococci (9%), group B streptococci (8%), *Klebsiella pneumoniae* (6%), *Staphylococcus aureus* (3%), *Proteus mirabilis* (2%), *Enterobacter* spp.(2%), and sporadically *Proteus vulgaris*, Gram positive cocci, *Citrobacter freundii*, and *Serratia Rubidea* (together less than 4%). When defined as any leukocyte excretion, the prevalence of leukocyturia was 66% in bacteriuric women and 34% in nonbacteriuric women, and when defined as five or more leukocytes per high power field the prevalence rates were 15%

Table 1 Baseline characteristics of all study participants, women with diabetes mellitus (DM) type 1 and women with DM type 2

Baseline characteristic	n	All women (n = 644)	Women with DM type 1 (n = 296)	Women with DM type 2 (n = 348)
Asymptomatic bacteriuria	644	110 (17%)	36 (12%)	74 (21%)
Age (year)	644	51.1 ± 15.2	41.1 ± 13.1	59.6 ± 11.2
Duration DM (year)	640	14.6 ± 11.5	20.0 ± 12.7	9.9 ± 7.6
≥ 1 UTI in last year before study enrollment	643	116 (18%)	43 (15%)	73 (21%)
Body Mass Index (BMI) (kg/m²)	644	27.4 ± 5.5	24.7 ± 3.8	29.6 ± 5.7
Hypertension	642	320 (50%)	81 (28%)	239 (69%)
Hemoglobin A1c (%)	643	8.5 ± 1.6	8.4 ± 1.4	8.5 ± 1.7
Creatinine clearance (ml/min)	644	95 ± 34	99 ± 29	92 ± 37
No. patients with peripheral neuropathy	587	189 (32%)	69 (26%)	120 (38%)
No. patients with macrovascular complications	643	138 (21%)	34 (12%)	104 (30%)
No. patients with retinopathy	631	193 (31%)	110 (38%)	83 (25%)
No. patients with				
microalbuminuria	606	98 (16%)	32 (11%)	66 (21%)
macroalbuminuria	606	31 (5%)	12 (4%)	19 (6%)

Values are given as mean ± standard deviation, or as number (and percentage).

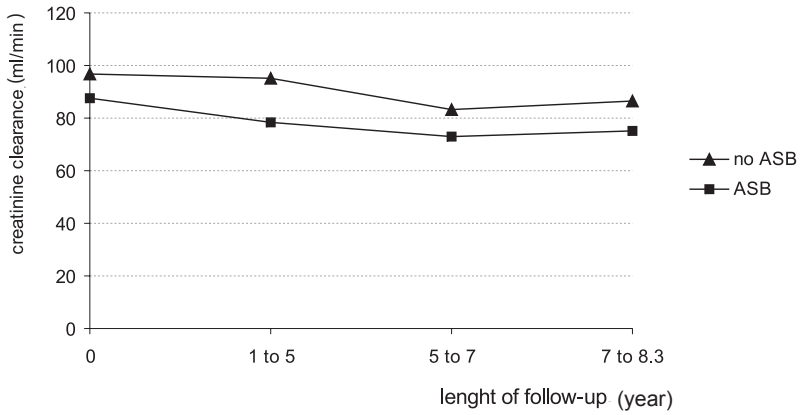
and 3%, respectively. Women with ASB had a significantly shorter follow-up than women without ASB; therefore all further analyses were corrected for the length of follow-up.
Four to six urine specimens were cultured within a time frame of approximately two years from 98 women (79 women with DM type 1 and 19 women with DM type 2). Of these, 56 women (57%) had no ASB on any occasion, five women (5%) showed persistent ASB, and 37 women (38%) had transient and/or recurrent ASB. *E. coli* was cultured from 22 of the 23 urine specimens collected from the women with persistent ASB.
From a total of 139 women a urine sample was collected at study endpoint or at least three years after the first urine culture, after a mean follow-up period of 5.3 ± 1.4 years. Women with ASB at baseline had an almost eight-fold increased risk of having ASB at this point compared to women who were nonbacteriuric at baseline (6 of 14 women (43%) with ASB versus 11 of 125 women (9%) without ASB at baseline; OR 7.7, 95% CI 1.9–31.0, p = 0.004, after adjusting for age and length of follow-up). In five of six women who had ASB on both occasions, *E. coli* was cultured from both urine samples.

Renal function and hypertension

The creatinine clearance decreased from 87 ml/min at baseline to 76 ml/min at study endpoint in diabetic women with ASB, and from 97 ml/min to 88 ml/min in those without ASB (*Figure 1*). In the univariate analysis, ASB was associated with a higher relative decrease in creatinine clearance (14 ± 22% and 9 ± 23% in women with versus women without ASB, respectively, p = 0.03), but not with the absolute decrease in creatinine clearance (12 ± 19 ml/min and 9 ± 20 ml/min, respectively, p = 0.12). In *Table 2* the possible risk factors for a creatinine clearance below 60 ml/min (a moderately decreased renal function)[2], including ASB and a history of UTIs are given. In the univariate analysis age, the length of follow-up, the duration of DM and microalbuminuria were identified as possible confounding factors when studying the influence of ASB on renal function development. Therefore, a multivariate analysis was done, according to age strata, and including the length of follow-up, duration of DM, and microalbuminuria at baseline. In the multivariate analysis no association was found between ASB and the relative or the absolute decrease in creatinine clearance. Also when women with DM type 1 and those with DM type 2 were analyzed separately, no association was found (data not shown). Finally, also no association with a faster decline in renal function was found when only the urines with *E. coli* as cultured microorganism were included in the analysis (data not shown).
At baseline, 50% of the total study group was diagnosed as having hypertension. After excluding these women and the women with missing values for hypertension at endpoint (n = 4), the remaining cohort consisted of 318 women. A total of 39 women (12%) of these 318 women had ASB. Women with ASB developed hypertension more often than women without ASB (54 versus 37%, p = 0.045). For

women with DM type 1 and type 2 separately, the difference was not statistically significant ($p > 0.1$). In the multivariate analysis, including age, duration of DM, and length of follow-up, the association between ASB and hypertension disappeared ($p > 0.2$); a higher age was the strongest predictor for the development of hypertension.

Figure 1 Development of the renal function of diabetic women with and without ASB



The percentages of patients, that were seen at each time point were 26% during 1 to 5 years of follow-up, 23% during 5 to 7 years of follow-up, and 51% during 7 to 8.3 years of follow-up.

Table 2 Risk factors (at baseline) for a creatinine clearance below 60 ml/min after six years follow-up, according to age categories, among a cohort of 644 women with diabetes mellitus (DM); univariate analysis[1]

	Age 18 to 36 years			Age 37 to 55 years			Age 56 to 75 years		
	creatinine clearance (ml/min)			creatinine clearance (ml/min)			creatinine clearance (ml/min)		
	≥60 (n = 136)	<60 (n = 4)	OR	≥60 (n = 197)	<60 (n = 24)	OR	≥60 (n = 151)	<60 (n = 132)	OR
Asymptomatic bacteriuria	14 (10%)	1 (25%)	2.9	24 (12%)	3 (13%)	1.0	30 (20%)	38 (29%)	1.6
≥ 1 UTI previous year (n = 643)	21 (15%)	1 (25%)	1.2	42 (21%)	5 (21%)	1.0	26 (17%)	21 (16%)	1.1
DM: type 1	122 (90%)	4 (100%)	–	107 (54%)	13 (54%)	–	25 (17%)	25 (19%)	–
type 2	14 (10%)	0 (0%)	–	90 (46%)	11 (46%)	–	126 (83%)	107 (81%)	–
Duration DM (year) (n = 640)	12 ± 8	23 ± 8	–	15 ± 12	22 ± 13[2]	–	13 ± 11	16 ± 14[2]	–
Creatinine clearance (ml/min)	121 ± 30	59 ± 31[2]	–	107 ± 31	65 ± 22[2]	–	88 ± 25	64 ± 17[2]	–
HbA1c (%) (n = 643)	8.1 ± 1.3	9.7 ± 1.5	–	8.5 ± 1.7	8.8 ± 1.9	–	8.6 ± 1.5	8.5 ± 1.7	–
Hypertension (n = 642)	24 (18%)	2 (50%)	4.6	74 (38%)	14 (58%)	2.3	109 (72%)	97 (74%)	1.1
Microalbuminuria (n = 606)	20 (15%)	2 (67%)[2]	11.2	26 (14%)	13 (57%)[2]	8.2	27 (20%)	41 (34%)[2]	2.1
Macroalbuminuria (n = 606)	3 (2%)	1 (33%)[2]	21.5	4 (2%)	9 (39%)[2]	29.9	5 (4%)	9 (8%)	2.2

Values are given as mean ± standard deviation, or as number of patients (and percentage); [1] the total number of patients is 644 unless otherwise stated; [2] $p < 0.05$.

Discussion

In contrast to our previous publication after 18 months follow-up,[11] in this study no association was found between ASB and a decline in renal function or the development of hypertension in women with DM type 1 or DM type 2 during a mean follow-up of six years. As shown, women with ASB at baseline had a lower creatinine clearance at study endpoint, a faster relative decrease in creatinine clearance and hypertension more often when compared univariate to women without ASB. However, the differences were mainly explained by differences in age and duration of DM and all differences disappeared in the multivariate analyses.

Several authors have shown that the prevalence of ASB in diabetic women is increased when compared to nondiabetic women.[5,13] In this study we report an overall prevalence of ASB of 17%, which is in agreement with previous findings in diabetic women, and higher than can be expected in women without DM.[14] This is somewhat lower compared to our previous study, because only 66% of women overlapped and a slightly different definition of ASB was used.[5,15] Despite the increased prevalence, to our knowledge no large cohorts with diabetic women have been followed long enough to prospectively study the consequences of ASB on renal function in this patient group. As mentioned in the introduction, we previously described the consequences of ASB during a follow-up period of 18 months, in a cohort partly overlapping the cohort described here.[11] During this short follow-up period of 18 months, women with DM type 2 and ASB had an increased risk of developing a symptomatic UTI. No increase was seen in the development of secondary complications (retinopathy, neuropathy, and micro- and macrovascular diseases) between diabetic women with versus those without ASB. In women with DM type 2, no differences in renal function development were seen between bacteriuric and nonbacteriuric participants. However, women with DM type 1 and ASB showed a tendency toward a faster decline in renal function than women without ASB (relative increase in serum creatinine level after 18 months was 4.6% and 1.5%, respectively), and therefore we prolonged the follow-up period. In a Polish study, also no differences in the incidence of pyelonephritis, hypertension, and creatinine levels were found between 53 diabetic patients with and 54 patients without ASB after 14 years of follow-up.[16] However, both men and women were included in this study, and only 25 cases and 24 controls completed the follow-up.

As we described before,[5] diabetic women with ASB are characterized by a longer duration of diabetes with the presence of secondary complications such as micro- or macroalbuminuria. In the present study, women with DM and ASB already had a lower creatinine clearance at baseline (87 and 97 ml/min in women with versus women without ASB, respectively, $p = 0.01$; *Figure 1*). It is possible that a longer duration of DM and subsequent diabetic nephropathy are associated with a higher vulnerability for bacterial adherence and with an inadequate local immune response in the urinary tract. In this study the prevalence of leukocyturia in diabetic women with ASB was only 15%. This might be due to the lower urinary cytokine excretion

which is correlated with a lower urinary leukocyte number and is lower in diabetic compared to nondiabetic patients with ASB as we have demonstrated before.[17] Others showed that certain *E. coli* strains stop expressing adherence factors like type 1 and P fimbriae once they have established bacteriuria.[18] Adherence of these fimbriae to uroepithelial cells is the trigger for the local urinary immune response. Therefore, the eventual result can be the persistence of ASB due to a defective clearance of bacteria, but without triggering an inflammatory response. This can also explain why ASB can persist without resulting in symptoms of a UTI and without renal damage. The results of the present study have led to the rejection of our hypothesis that ASB in itself can lead to a decline in renal function, either in women with DM type 1 or in women with DM type 2. Therefore it is not likely that treatment of ASB will lead to a decrease in the incidence of diabetic nephropathy or end-stage renal disease. This is in accordance with a recent study among diabetic women with ASB, in which a comparison was made between women who received antibiotic therapy and women who received placebo. In this study no difference was seen in serum creatinine levels after a mean follow-up of two years.[8]

In nondiabetic individuals, a correlation between ASB and hypertension has been shown by some authors, but not by others, as reviewed before.[14,19] We found a high prevalence of hypertension in our cohort. Hypertension was defined as a blood pressure above 140/90 mm Hg or the use of antihypertensive medication. Since the present guidelines use more strict criteria to classify patients to have hypertension the number of patients with hypertension can even be underestimated. On the other hand, an overestimation is also possible,because some individuals might have been treated for instance with angiotensin-converting enzyme inhibitors because of microalbuminuria. However, it is unlikely that this will effect the associations between ASB and hypertension.

The results of our study are strengthened by the prospective design, the large sample size and the long follow-up.

Our study has several limitations. A potential limitation is our reliance on one culture to diagnose ASB. We made the assumption that however bacteriuria might be transient in a proportion of the bacteriuric study subjects, bacteriuria at one point reflects a higher susceptibility to recurrent and persistent bacteriuria in general, even after antimicrobial therapy. Our findings on the follow-up cultures as described above, as well as previous findings of others,[20] are supportive with this assumption. In this study, especially bacteriuria with *E. coli* seemed to persist. It has been described before that type 1-fimbriated *E. coli* can invade the superficial epithelial cells that line the luminal bladder surface and subsequently replicate, establishing a persistent bacterial reservoir within the bladder mucosa.[21] Furthermore, we have demonstrated with molecular identifying techniques that *E. coli* can persist in the urine of diabetic women without leading to symptoms during a period of four months.[22]

Another limitation of our study can be the use of the creatinine clearance calculated by the Cockcroft-Gault formula as an estimation of the GFR. The accuracy of the creatinine clearance is limited by the fact that creatinine is not only filtered across

the glomerulus but also excreted by the tubulus. If the GFR falls, the creatinine secretion is enhanced, leading to an overestimation of the GFR. However, keeping this in mind, the creatinine clearance, as well as the use of the Cockcroft-Gault formula to calculate it, were extensively evaluated and found reliable by several others before.[2] Furthermore, we do not have complete information about the antimicrobial treatment for UTIs during the total follow-up period. The results of the 18-month follow-up period showed no differences in antimicrobial treatment between DM type 1 women with and without ASB (12% and 13% respectively), but for women with DM type 2 with and without ASB these percentages were 29% and 15% respectively. However, no association between antimicrobial use and renal function decline could be demonstrated in this group.[11]

A high percentage (70%) of the women with DM type 2 used insulin alone or in combination with oral hypoglycemic medication. In addition, they were mainly recruited from outpatient departments, and only a minority came from the offices of general practitioners, whereas the latter treat a remarkable part of patients with DM type 2. It is therefore questionable whether our results can be extrapolated to all women with DM type 2. However, the most important endpoints, renal insufficiency and hypertension, have a higher prevalence in the studied population than in the population from the general practitioners. Therefore, an association between ASB and these two conditions is not to be expected in the total patient group if it does not exist in a selection of patients with a more complicated course of their diabetes.

In conclusion, in our opinion we can reject our hypothesis that ASB will lead to renal function deterioration in women with DM, because we found no difference in renal function development in women with DM type 1 or DM type 2 after a mean follow-up of six years. Also the incidence of hypertension was not increased when comparing women with versus women without ASB. Therefore, in our opinion, screening for and subsequent treatment of ASB are not indicated in patients with diabetes.

Acknowledgements

This study was supported by a grant from the Dutch Diabetes Fund (number 95.123). We would like to thank the participating physicians E.W.M.T. ter Braak, M.C. Castro Cabezas, J.Th. Collet, T.W. van Haeften, P.C. Ligtenberg-Oldenburg, H.W. de Valk, H.E. Westerveld, and P.M.J. Zelissen (University Medical Center Utrecht), B. Bravenboer, and M.J.L. Camps (Catharina Hospital Eindhoven), J.B.L. Hoekstra (Academic Medical Center, Amsterdam), K.P. Bouter (Jeroen Bosch Hospital, 's Hertogenbosch), W.M.N. Hustinx (Diakonessenhuis Utrecht), the general practioners C.L.M. Appelman, C.P. Bouter, W.H. Eizenga, Y.W.M.Gresnigt, W. van der Kraan, and M.E. Numans (Utrecht) and G. Ijff (Amsterdam), the medical microbiologists J. Verhoef (Utrecht), A.R. Jansz (Veldhoven), and R.J.A. Diepersloot (Utrecht), and the laboratory technician E.C. Brouwer (Utrecht).

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Twelve

Summary and General Discussion

Samenvatting

Dankwoord

Curriculum Vitae

Urinary tract infections (UTIs) are among the most common bacterial infections acquired by humans. Women with diabetes mellitus (DM) have a higher risk of UTIs compared to women without DM,[1-4] which also tend to have a more complicated course.[5,6] UTIs can be divided in categories according to different criteria, for instance in upper or lower UTIs, complicated or uncomplicated UTIs, and symptomatic or asymptomatic UTIs. The studies in this thesis mainly concern asymptomatic bacteriuria (ASB), specifically in women with DM.

ASB is defined as the presence of 10^5 colony forming units (cfu) of one or two uropathogens per milliliter of urine from a patient without symptoms of a UTI. Despite the high prevalence of ASB in women with DM,[4] there are still aspects on this condition that have not (enough) been studied before. We focused on three aspects of ASB. First, what is the pathogenesis of the increased prevalence of ASB in women with DM? Is this due to a difference in the host or in the microorganism? Second, how can ASB be diagnosed, besides by the classical urine culture? And finally, must we be alerted by the higher prevalence in this patient group, in other words, what are the consequences of ASB in women with DM? The studies described in this thesis addressed these questions and the results are discussed below and placed in the context of current literature.

1. Pathogenesis of ASB in women with DM

As summarized in **Chapter 2**,[7] bacteria colonizing the perineum and vagina can enter the bladder where they can adhere to uroepithelial cells lining the bladder mucosa, or even further ascend to the kidneys. Whether an infection is established depends on several factors that can be divided in host related and pathogen related factors, of which some are specific for diabetic patients. The increased frequency of UTIs in diabetic patients is likely due to a combination of these factors. Possible host related mechanisms which have been studied before are glycosuria, defects in neutrophil function, and differences in cytokine secretion.[7] Furthermore, different risk factors have been identified, such as macroalbuminuria, diabetic neuropathy, sexual intercourse and a history of UTIs.[4,8] Poor glycemic control does not seem to be an independent risk factor.[3,8] Pathogen related factors specific for different patient groups are less known. Microorganisms possess a variety of virulence factors, for example type 1 and P fimbriae. Others have shown that *Escherichia coli* (the most prevalent uropathogen) isolated from patients with a symptomatic UTI express fewer virulence factors when the affected patient has an abnormal urinary tract or is immunocompromised (including diabetic patients) than those isolated from healthy controls.[9] This suggests that in the presence of a patient specific factor that predisposes for a (urinary) tract infection, the causative microorganism can cause an infection while being less virulent. However, this appears to be different for ASB, since the number of virulence factors in *E. coli* isolated from the urine of diabetic women with ASB was comparable to the results found in noncompromised patients with ASB.[10]

The adherence of the microorganisms to glycoprotein receptors (uroplakins) on the

uroepithelial cells is an essential step in the pathogenesis of UTIs, and is mediated by type 1 fimbriae.[11] We found that the adherence of type 1-fimbriated *E. coli* to uroepithelial cells of women with DM is increased, compared to the adherence to uroepithelial cell of women without DM, as described in **Chapter 3**.[12] It seems likely that this increased adherence plays a role in the pathogenesis of UTIs in women with DM. The adhesive part of type 1 fimbriae is the FimH adhesin, located at the distal end of the pilus. Others have demonstrated in animal studies that Fim(C)H-adhesin-based vaccination could prevent *E. coli* bacteriuria.[11,13] In **Chapter 4** we describe the *in vitro* studies showing that FimCH antiserum also inhibits the adherence of *E. coli* to cells voided by diabetic women.[14] Moreover, we show that the *fimH* sequence of *E. coli* strains isolated from women with DM is highly homologous to the known *fimH* sequence of *E. coli* from nondiabetic patients. Therefore, if a difference in the "adherence step" is the cause of the higher prevalence of ASB in women with DM, it is most probably due to a host specific factor. The difference between diabetic and nondiabetic women might be in the uroplakins on the uroepithelium. As mentioned above, these receptors for type 1 fimbriae are glycoproteins, and carry mannose containing carbohydrates.[15] Changes in cell surface carbohydrates can directly result in an altered susceptibility to microbial infections. It has already been demonstrated that the carbohydrate structures on serum proteins are modified in patients with DM.[16] It is therefore possible that type 1-fimbriated *E. coli* show an increased adherence to uroplakins due to an altered carbohydrate composition on these proteins. This composition change might be influenced by glycosuria.

An alternative explanation for the difference between women with and women without DM is an altered interaction between the diabetic host and the microorganism in which an unknown host factor influences the phase-variation of the type 1 fimbriae. Phase-variation refers to the process in which the promoter of the *fimA*-gene (that encodes the major subunit of type 1 fimbriae) is switched from the on- to the off-position and vice-versa. Only in the on-position the fimbrial gene is transcribed, resulting in the phenotypic expression of type 1 fimbriae.[17] One could hypothesize that in diabetic patients a larger percentage of the inoculated *E. coli* is being switched on by any cause, resulting in more adhering bacteria that are prevented from being voided. This was not confirmed by previous *in vitro* results: only 59% of the *E. coli* strains isolated from diabetic patients with ASB was phenotypically positive for type 1-fimbriae.[10] But it is important to realize that the phenotype of voided uropathogens, as used in this study, can be different from the *in vivo* situation in which the uropathogens are adhering to the uroepithelial cells.

We also studied the resistance rates of uropathogens isolated from the urine of diabetic patients with ASB, as described in **Chapter 5**.[18] When we consider DM as an immunocompromising disease and resistance as a virulence factor, we could expect to isolate less virulent uropathogens with a lower resistance rate from women with DM. On the other hand, a higher resistance rate could be expected in uropathogens of diabetic patients because of the increased incidence of UTIs in these patients, and the subsequent increased use of antibiotics. We analyzed the resistance of *E. coli* strains (n = 135) isolated from the urine of diabetic women

with ASB and compared those with routine isolates (n = 5907) from the female population in the same area. The resistance rates of *E. coli* isolated from diabetic women and routine isolates of *E. coli* to trimethoprim-sulfamethoxazole were 19% and 23%, respectively, to amoxicillin 16% and 32%, to nitrofurantoin 1% and 3%, to ciprofloxacin 0% and 4%, to ofloxacin 0% and 5%, and to norfloxacin 1% and 4%.[18] Although the comparison group was not ideal, the large number of isolates that were studied at least allowed us to conclude that diabetes in itself is not associated with higher resistance rates.

In conclusion, the increased prevalence of ASB and incidence of UTIs in diabetic compared to nondiabetic women is most likely due to a difference in the diabetic host and not in the causative microorganism.

Future research must show whether the difference in the host is the result of an altered or increased number of uroepithelial cell receptors on the cells of patients with DM. Furthermore, it must be investigated if a possible altered cell receptor is the result of an altered carbohydrate composition of this glycoprotein, and whether this can be influenced by a better regulation of the diabetes.

2. Diagnosis of ASB

In **Chapter 6 and Chapter 7** we described two alternative ways to diagnose ASB. In **Chapter 6** we demonstrated a scoring system based on two variables routinely obtained in daily clinical practice (duration of DM and glycosylated hemoglobin for women with DM type 1, and age and the number of symptomatic UTIs in the previous year for women with DM type 2) that in combination with a urinary leukocyte count accurately predicts the presence of ASB in women with DM (with an area under the receiver operating characteristic curve higher than 0.75).[19] Others have described a comparable scoring system for symptomatic lower UTIs.[20] But it was notable that, even without the addition of the urinary leukocyte count, a reasonable model could be designed for ASB also, as it is an obligatory asymptomatic condition. Saving the costs of a urine culture, it provides us with a possible screening tool for ASB in this patient group. However, whether screening is warranted is still up for debate (see below).

In **Chapter 7** we described the development and validation of a real-time Polymerase Chain Reaction (PCR)-based assay to diagnose *E. coli* bacteriuria.[21] Our primary interest was to develop a test to detect bacteriuria in long-time stored urine. We had the unique opportunity to use an extensive population-based database with a long follow-up of which the participants' urine had been stored since the 1970's. These urine samples could not be tested reliably with routine culturing methods, and also a leukocyte count was not possible since an unknown percentage of the leukocytes would have been destroyed during storage. The great advantage of the real-time PCR was its usage of bacterial DNA as it remains untouched in time. Real-time PCRs are used more and more in clinical setting to test an increasing number of infections with the advantages of often high specificity and specificity as well as being faster than most culturing methods. The sensitivity and specificity of the presented real-

time PCR for *E. coli* bacteriuria were 92% and 87%, respectively, while the assay required three hours. We acknowledge that the test has restrictions for clinical use. For instance, bacteriuria with other uropathogens will remain undetected and the test does not provide a resistance pattern. Further development of the test possibly can extent its possibilities, however this was beyond our purpose. For research settings, the test has provided us with a useful tool.

3. Consequences of ASB in women with and women without DM

If ASB is diagnosed for whatever reason, physicians, whether or not asked to by their patients, are inclined to treat this infection. However, this might lead to unwanted side-effects, and contribute to the increasing antimicrobial resistance among uropathogens. Realizing that there is a big chance that the ASB recurs after antimicrobial treatment, as shown by Harding et al.,[22] one should reconsider the indication of antimicrobial treatment. And that depends mainly on the potential (long-term) consequences of ASB and whether these would be ameliorated by intervention.

ASB in nondiabetic patients has been associated with recurrent symptomatic UTIs, hypertension, renal function deterioration, complications of pregnancy, and increased mortality.[23,24] However, study results have been contradictory, and no study has convincingly shown that bacteriuria in itself can cause severe health damage that can be prevented by antimicrobial intervention. Therefore, guidelines do not advise screening on or treatment of ASB except for some well defined patient groups.[25] Hypertension and renal failure are both important health problems in nondiabetic as well as in diabetic patients leading to high morbidity and medical costs.[26-29] We intended to study the possible effects of ASB on the development of hypertension and on renal function in order to answer the question whether ASB needs treatment.

In **Chapter 8 and Chapter 9** we investigated whether *Escherichia coli* bacteriuria is associated with a decline in renal function, the development of end-stage renal failure, or with hypertension in a healthy female population after a long-term follow-up.

To address the relation between *E. coli* bacteriuria and renal function development (**Chapter 8**), a full cohort analysis was performed for women who participated in two population based studies that took place in the (surroundings of) the city of Utrecht, the Netherlands; the first in the 1970's to 1980's (the baseline cohort), and the second in the 1990's (the follow-up cohort). In the final cohort analysis 490 women were included with a mean follow-up of 11.5 ± 1.7 years. It was shown that 48 women (10%) had *E. coli* bacteriuria at baseline (diagnosed using stored urine samples by the real-time PCR described in **Chapter 7**). After 11.5 years the mean creatinine clearance for women with versus women without bacteriuria was 87 ± 21 and 85 ± 18 ml per minute, respectively. *E. coli* bacteriuria at baseline was not associated with creatinine levels, adjusted for age and weight ($p = 0.71$). In addition, a nested case-control study was performed; cases were all women who

underwent kidney-replacing therapy in the Netherlands between participation in the baseline cohort and May 2002 (n = 49, mean duration from baseline until kidney-replacing therapy of 13.8 ± 7.4 years). The prevalence of *E. coli* bacteriuria was 14% among both cases and the controls. The odds ratio for the development of renal failure in the presence of *E. coli* bacteriuria at baseline, corrected for age, was 1.1 (95% confidence interval (CI) 0.4–2.8, p = 0.86). We therefore concluded that *E. coli* bacteriuria was not associated with a decline in renal function or with the development of end-stage renal failure in a population of generally healthy adult women during 12 to 14 years of follow-up.

In **Chapter 9** we analyzed the same population with the development of hypertension as primary endpoint. Interestingly, we found that women with *E. coli* bacteriuria were at increased risk for the development of hypertension (OR 3.1, 95% CI 1.6–6.0). This association has been suggested before,[30,31] but no prospective study has convincingly shown that bacteriuria in itself leads to hypertension. The underlying mechanism of this finding is not clear. Beforehand, we hypothesized that bacteriuria could lead to renal damage and subsequently to hypertension, but, as discussed above, we did not find a difference in creatinine clearance between bacteriuric and nonbacteriuric women in the same cohort. It is possible that hypertension occurs before renal function deterioration becomes apparent in a reduction in creatinine clearance (as described for example in chronic glomerulonephritis).[32] An alternative and more likely explanation is that both bacteriuria and hypertension are found more frequently among individuals with comorbidity or that they share a same (unknown) cause. This is supported by the higher prevalence of bacteriuria among women who used antihypertensive drugs at baseline. In conclusion, we found a strong association between bacteriuria and hypertension in a population of healthy women. Given the importance of hypertension further study is warranted on the nature of this.

Previous studies by our study group demonstrated that women with DM type 1 and ASB showed a tendency to a faster decline in renal function than women without ASB after 18 months follow-up.[33] However, the difference did not reach statistical significance, and was not apparent in women with DM type 2. Since DM is one of the main causes of renal failure leading to high morbidity and economic burden,[28] our previous results made us eager to reject or confirm the possible association between ASB and renal function deterioration. Before, others could not find a difference in creatinine levels between bacteriuric and nonbacteriuric individuals, but the results were not conclusive because of the small size of the study group,[34] or the short follow-up.[22]

To study the long-term consequences of ASB on renal function in diabetic women, our original cohort of diabetic women was enlarged and the follow-up period was prolonged, as discussed in **Chapter 10** (in a reaction to the above mentioned study by Harding et al.).[22,35] This resulted in the prospective multicenter study that is described in **Chapter 11**. Women with ASB at baseline were compared to women without ASB for differences in renal function development and also for the incidence of hypertension. A total of 644 adult women with DM type 1 (n = 296) or type 2

(n = 348) were included, of whom 110 women (17%) had ASB at baseline. The mean duration of follow-up was 6.1 ± 1.9 years, ranging from 1.0 to 8.3 years. In diabetic women without ASB, the creatinine clearance decreased from 97 ml/min at baseline to 88 ml/min at study endpoint; in diabetic women with ASB the creatinine clearance decreased from 87 to 76 ml/min. In the multivariate analyses, according to age strata, and including the length of follow-up, duration of DM, and microalbuminuria at baseline, no association was found between ASB and the relative or the absolute decrease in creatinine clearance. Also when women with DM type 1 and women with DM type 2 were analyzed separately, no association was found between ASB and renal function. Women with ASB developed hypertension more often than women without ASB (54 versus 37%, p = 0.045), but in the multivariate analysis, including age, duration of DM, and length of follow-up, the association between ASB and hypertension also disappeared (p > 0.2). We concluded that ASB does not lead to a faster decline in renal function or to the development of hypertension in women with DM type 1 or type 2 after six years of follow-up.

In the Netherlands nearly all patients with DM type 1 visit an outpatient department for their diabetes regulation, and we tried to include an unselected part of these women for our studies. However, the women with DM type 2 who participated in our study were also recruited mainly from outpatient departments, and only a minority came from the offices of general practitioners, whereas the latter treat a remarkable part of patients with DM type 2. It is therefore questionable whether our results can be extrapolated to all women with DM type 2. However, the most important endpoints, renal insufficiency and hypertension, have a higher prevalence in the studied population than in the population from the general practitioners. Therefore, an association between ASB and these two conditions is not to be expected in the total patient group if it does not exist in a selection of patients with a more complicated course of their diabetes. Besides that, we only included women in our study. UTIs in men have a different pathology compared to UTIs in women, and the prevalence is much lower. Therefore the conclusions can not be extrapolated to the male diabetic population.

It remains intriguing how such a high percentage of diabetic women can have ASB without serious consequences. The bacteria seem to remain in the bladder of diabetic patients without triggering an immune response. In general, the adherence of uropathogens to uroepithelial cell receptors activates different transmembrane signaling pathways, resulting in the release of chemokines (for instance interleukin-8 [IL-8] and IL-6) and subsequently to the recruitment of neutrophils to the bladder.[36] Neutrophils are essential for the clearance of the bacteria. The urinary secretion of IL-8 and IL-6 is lower in diabetic women than in nondiabetic women,[37] despite the fact that more bacteria adhere to the uroepithelial cells of women with DM. This impaired secretion of cytokines might contribute to a diminished clearance of bacteria. Animal studies have shown that type 1-fimbriated *E. coli* can invade the uroepithelial cells, were they can replicate and form intracellular reservoirs.[38] Because of the lower urinary cytokine concentrations in women with DM, we can hypothesize that through the increased adherence and impaired inflammatory

response, the bacteria more easily invade the uroepithelial cells. Thereby the innate host defenses may be evaded,[39] while ASB persists without causing tissue damage.

4. Should ASB in women with DM be treated?

As mentioned above, no study has convincingly shown an association between ASB and serious health damage in otherwise healthy nonpregnant patients.[25,40] Whether this was also true for women with DM was insufficiently studied. In our view, the main issue was raised by our previous studies showing that women with DM type 1 and ASB had a faster (but not statistically significant) decline in renal function than those without ASB after 18 months follow-up (relative increase in serum creatinine 4.6% versus 1.5%, $p = 0.2$).[33] In the same study, ASB did not increase the risk of developing retinopathy, peripheral neuropathy or macrovascular diseases in women with DM type 1 or type 2. Although 18 months follow-up is somewhat short for a definite conclusion on this matter, the figures did not even show a tendency to the occurrence of more diabetic complications in the bacteriuric women. It was however shown that women with DM type 2 who had ASB at baseline had an increased risk of developing a symptomatic UTI during follow-up (17% without ASB versus 27% with ASB, $p = 0.02$). In bacteriuric women with DM type 1 there was no increased incidence of symptomatic UTIs.[33] One might argue therefore that women with DM type 2 and ASB should receive treatment. Arguments pro treatment would be to spare the patient the inconvenience of UTI symptoms and to prevent the possible complications of a symptomatic UTI. However, a recent randomized placebo-controlled trial showed that antimicrobial treatment of ASB in diabetic women did not reduce the time to a first symptomatic episode, the overall rate of symptomatic UTIs, or the hospitalization rate. The authors showed the difficulty in keeping the patients free of bacteriuria despite antimicrobial treatment.[22] Therefore, although we found an association between ASB in women with DM type 2 and the occurrence of symptomatic UTIs, in our opinion it is not an indication for treatment. In accordance with our findings, the authors of the trial on antimicrobial treatment found no difference in creatinine levels between the women in the placebo and the women in the antimicrobial-group after approximately two years follow-up.[22] The results of our study have led to the rejection of our hypothesis that ASB will lead to renal function deterioration in women with DM (type 1). Therefore, in our opinion, screening for and subsequent treatment of ASB are not indicated in women with DM type 1 or type 2.

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Twelve

Summary and
General Discussion

Samenvatting

Dankwoord

Curriculum Vitae

Bacteriurie is de aanwezigheid van bacteriën in urine. Uropathogenen zijn bacteriën die in potentie een urineweginfectie kunnen veroorzaken. De meeste studies in dit proefschrift gaan over asymptomatische bacteriurie. Asymptomatische bacteriurie wordt gedefinieerd als de aanwezigheid van uropathogenen in een vastgestelde hoeveelheid (10^5 kolonie vormende eenheden) per milliliter urine van een patiënt zonder klachten van een urineweginfectie (bijvoorbeeld pijn bij het plassen). *Escherichia coli* (*E. coli*) is het meest voorkomende uropathogeen.
Vrouwen met diabetes mellitus hebben vaker urineweginfecties dan vrouwen zonder diabetes. Daarnaast kunnen urineweginfecties bij deze patiëntengroep vaker gecompliceerd verlopen en zijn ze mogelijk moeilijker te behandelen. Uit voorgaand onderzoek is gebleken dat bij ongeveer een kwart van de vrouwen met diabetes asymptomatische bacteriurie kan worden vastgesteld, drie tot vier keer vaker dan bij vrouwen zonder diabetes. De oorzaak van dit verschil is nog niet geheel opgehelderd, evenmin als de mogelijke gevolgen van asymptomatische bacteriurie op de lange termijn. Er zijn twee vormen van diabetes mellitus. Diabetes mellitus type 1 ontstaat op jonge leeftijd en de patiënten zijn afhankelijk van insuline. Diabetes mellitus type 2 ontstaat vaak op oudere leeftijd en kan, zeker in het begin, vaak met tabletten worden behandeld.

Dit proefschrift is verdeeld in twee delen waarin verschillende onderzoeksvragen aan bod komen. Het eerste deel (**Hoofdstuk 2** tot en met **Hoofdstuk 7**) betreft de pathogenese en de diagnostiek van asymptomatische bacteriurie bij vrouwen met diabetes mellitus. Wat is de oorzaak van de verhoogde incidentie? Komt dit door een verschil in de gastheer, in dit geval de vrouwen met diabetes, of door een verschil in micro-organisme, het uropathogeen? Zijn er naast de urinekweek nog andere, mogelijk snellere, manieren om asymptomatische bacteriurie te diagnosticeren? Het tweede deel (**Hoofdstuk 8** tot en met **Hoofdstuk 11**) bevat studies naar de gevolgen van asymptomatische bacteriurie, zowel bij vrouwen zonder als vrouwen met diabetes. Hebben vrouwen met asymptomatische bacteriurie een hoger risico op het ontwikkelen van hypertensie (hoge bloeddruk), een snellere nierfunctieverslechtering of mogelijk zelfs nierfalen?

Hoofdstuk 2 geeft een samenvatting van de bestaande literatuur over urineweginfecties bij patiënten met diabetes mellitus. Aangezien urineweginfecties bij mannen een veel lagere incidentie hebben, zijn vrijwel alle studies gedaan bij vrouwen. Urineweginfecties ontstaan praktisch altijd via de opstijgende route. Uropathogenen, afkomstig van de darmflora, koloniseren het gebied van de anus tot de vagina, en migreren naar de blaas. Door verdunning en regelmatig uitplassen worden de bacteriën veelal weer geklaard. Uropathogenen beschikken echter over verschillende virulentiefactoren (ziekmakende factoren) waarmee zij dit kunnen voorkomen. In de pathogenese van lagere urineweginfecties spelen vooral type 1 fimbriae een grote rol. Dit zijn haarachtige structuren op het bacteriële celmembraan waarmee de bacteriën zich kunnen hechten aan receptoren op de blaascellen (adherentie). Of een urineweginfectie ontstaat,

hangt af van zowel gastheergerelateerde als uropathogeengerelateerde factoren. De verhoogde incidentie van asymptomatische bacteriurie bij diabetespatiënten wordt waarschijnlijk veroorzaakt door een combinatie hiervan. Mogelijke gastheerfactoren die eerder zijn bestudeerd zijn de aanwezigheid van glucosurie (suiker in de urine), verminderde functie van neutrofielen (witte bloedcellen) en een verminderde productie van cytokines (kleine eiwitten die belangrijk zijn bij de afweer). Bekende risicofactoren voor het krijgen van asymptomatische bacteriurie voor vrouwen met type 1 diabetes zijn een langere duur van de diabetes, perifere neuropathie (stoornissen in de zenuwgeleiding) en macroalbuminurie (eiwit in de urine, een symptoom van nierschade). Voor vrouwen met type 2 diabetes zijn dit hogere leeftijd, een recente symptomatische urineweginfectie en opnieuw macroalbuminurie. Net als bij vrouwen zonder diabetes is *E. coli* het belangrijkste uropathogeen, hoewel andere bacteriën relatief vaker voorkomen.
Daarnaast wordt in dit hoofdstuk verder ingegaan op de richtlijnen over de behandeling van urineweginfecties bij vrouwen met diabetes. Omdat onvoldoende bekend was over mogelijk nadelige gevolgen van asymptomatische bacteriurie, was de noodzaak tot behandeling van asymptomatische bacteriurie controversieel. Voor de behandeling van symptomatische lage urineweginfecties (blaasontstekingen) wordt over het algemeen een antibioticum geadviseerd waarmee een hoge weefselconcentratie kan worden bereikt (en niet alleen een hoge concentratie in de urine). De optimale duur van de behandeling is mogelijk langer dan bij patiënten zonder diabetes, namelijk zeven tot veertien dagen. De behandeling van pyelonefritis (nierbekkenontsteking) is bij patiënten met en zonder diabetes hetzelfde.
Zoals hierboven staat beschreven is de hechting van bacteriën aan blaascellen een belangrijke stap in de pathogenese van urineweginfecties. In **Hoofdstuk 3** worden de resultaten beschreven van een laboratoriumstudie waaruit blijkt dat de adherentie van *E. coli* (met type 1 fimbriae) aan blaascellen twee keer zo hoog is bij cellen afkomstig uit urine van vrouwen met diabetes in vergelijking met cellen van vrouwen zonder diabetes. Dit zou deels de verhoogde incidentie van urineweginfecties bij vrouwen met diabetes kunnen verklaren.
Er bestaan vele *E. coli*-stammen die van elkaar verschillen in bijvoorbeeld de aanwezigheid van en soort virulentiefactoren zoals type 1 fimbriae. Men zou kunnen hypothetiseren dat de urineweginfecties bij vrouwen met diabetes mellitus worden veroorzaakt door een andere selectie *E. coli*-stammen. Met de wetenschap dat type 1 fimbriae een belangrijke rol spelen bij het ontstaan van urineweginfecties hebben we in **Hoofdstuk 4** verschillende stammen afkomstig uit urine van vrouwen met en zonder diabetes met elkaar vergeleken. Hierbij bleek op genniveau dat er geen noemenswaardig verschil is tussen de sequenties van het FimH gedeelte dat codeert voor het hechtende gedeelte van type 1 fimbriae (zogezegd het grijphaakje waarmee ze aan de blaascel hechten). Dit maakt het onwaarschijnlijk dat de verhoogde hechting wordt veroorzaakt door een verschil in micro-organisme en pleit meer voor een gastheergerelateerde factor. Daarnaast wordt beschreven dat antistoffen tegen dit FimH gedeelte de adherentie van

E. coli aan blaascellen van vrouwen met diabetes verminderen, wat al eerder was vastgesteld bij cellen van vrouwen zonder diabetes mellitus.
Wereldwijd hebben we te maken met een stijging van de resistentie van bacteriën tegen antibiotica. In **Hoofdstuk 5** vergelijken we de resistentiegegevens van 135 *E. coli*-stammen gekweekt uit urine van vrouwen met diabetes met die van 5907 isolaten afkomstig uit urine van een doorsnee groep vrouwen uit dezelfde regio. Uit de resultaten kan worden geconcludeerd dat diabetes op zich niet geassocieerd is met een hogere resistentie.

De klassieke methode om vast te stellen of iemand een urineweginfectie heeft, is de urinekweek. Dit wordt over het algemeen beschouwd als de gouden standaard. Nadelen van de urinekweek zijn de kosten, het feit dat de uitslag vaak pas na 48 uur bekend is en het vereiste dat de urine relatief vers is. In **Hoofdstuk 6** wordt een model gepresenteerd waarmee het mogelijk is om met behulp van twee routinematig verkregen variabelen in combinatie met de telling van het aantal witte bloedcellen in de urine een goede voorspelling te geven of er sprake is van asymptomatische bacteriurie. De twee variabelen verschillen tussen vrouwen met type 1 en type 2 diabetes. Voor vrouwen met type 1 diabetes waren deze variabelen de duur van de diabetes en de waarde van het geglycosyleerde hemoglobine (een maat voor de diabetesregulatie). Voor vrouwen met type 2 diabetes waren dit de leeftijd en het aantal symptomatische urineweginfecties in het voorafgaande jaar. Een dergelijk model kan bijvoorbeeld gebruikt worden voor screening van grote groepen patiënten, in dit geval op het hebben van asymptomatische bacteriurie.
In **Hoofdstuk 7** staat de ontwikkeling van een tweede alternatief voor de urinekweek beschreven waarbij gebruik wordt gemaakt van het genetische materiaal van bacteriën in urine. Dit is gebaseerd op een zogenaamde real-time Polymerase Chain Reaction. Hierbij wordt een klein stukje DNA vermenigvuldigd dat specifiek is voor wat men wil opsporen, in dit geval *E. coli*. Hierbij wordt gebruik gemaakt van een gelabeld stukje DNA, de probe, dat tijdens het proces een fluorescentiesignaal afgeeft. Afgezet tegen een standaardlijn is het uiteindelijke resultaat een kwalitatief en kwantitatief diagnosticum voor de aanwezigheid van *E. coli* in urine. Omdat het hierbij niet van belang is of de *E. coli* nog leeft, is het mogelijk hiermee urinemonsters te testen die al geruime tijd zijn bewaard. Daarom is het voor ons een waardevolle test gebleken bij de onderzoeken die hieronder worden beschreven.

In het tweede deel van dit proefschrift staan patiëntgebonden onderzoeken beschreven naar de lange termijn gevolgen van asymptomatische bacteriurie bij zowel vrouwen zonder als vrouwen met diabetes mellitus.
Om te onderzoeken of *E. coli*-bacteriurie op de langer termijn leidt tot een snellere verslechtering van de nierfunctie of tot eindstadium nierfalen (gedefinieerd als het ondergaan van nierfunctievervangende therapie) werd een cohortanalyse gedaan onder vrouwen die deel hadden genomen aan twee populatiestudies die plaatsvonden in Utrecht en omgeving; de eerste van 1974 tot 1986 (het baseline

cohort) en de tweede van 1993 tot 1997 (het follow-up cohort). De resultaten staan beschreven in **Hoofdstuk 8**. In totaal werden 490 vrouwen geïncludeerd, met een gemiddelde follow-up duur van 12 jaar. Met behulp van de real-time Polymerase Chain Reaction die beschreven staat in Hoofdstuk 7 kon worden vastgesteld dat 48 vrouwen (10%) *E. coli*-bacteriurie hadden aan het begin van de studie. Na 12 jaar was de nierfunctie van de vrouwen met bacteriurie hetzelfde als die van de vrouwen zonder bacteriurie. Van het baseline cohort konden 49 vrouwen worden geïdentificeerd die na gemiddeld 14 jaar nierfunctievervangende therapie moesten ondergaan (de cases). Deze werden vergeleken met vier maal zo veel vrouwen afkomstig uit hetzelfde cohort die de controlegroep vormden. The prevalentie van *E. coli*-bacteriurie op baseline was voor beide groepen 14%. Daarmee was het risico voor een vrouw om nierfalen te ontwikkelen onafhankelijk van het hebben van bacteriurie op baseline (odds ratio 1,1, 95% betrouwbaarheidsinterval 0,4 – 2,8).

In **Hoofdstuk 9** wordt dezelfde populatie geanalyseerd met als eindpunt hypertensie (hoge bloeddruk). Opvallend was de bevinding dat vrouwen met *E. coli*-bacteriurie een duidelijk verhoogd risico hadden op de ontwikkeling van hypertensie (odds ratio 3,1, 95% betrouwbaarheidsinterval 1,6 – 6,0). De oorzaak van deze relatie is onduidelijk. De meest logische hypothese, namelijk dat bacteriën nierschade veroorzaken en dat pas in tweede instantie hypertensie ontstaat, wordt tegengesproken door de eerder genoemde bevinding dat de nierfunctie van vrouwen met en zonder bacteriurie gelijk bleef. Waarschijnlijker is dat bacteriurie en hypertensie beide vaker voorkomen bij vrouwen met een slechtere gezondheid of dat beide aandoeningen een (nog onbekende) risicofactor gemeen hebben.

Uit voorgaand onderzoek van onze studiegroep was gebleken dat vrouwen met type 1 diabetes en asymptomatische bacteriurie op baseline na 18 maanden follow-up een slechtere nierfunctie hadden dan vrouwen met type 1 diabetes zonder asymptomatische bacteriurie. Dit verschil was echter niet statistisch significant en gold niet voor vrouwen met type 2 diabetes. Zoals beargumenteerd in **Hoofdstuk 10** was dit echter wel reden om de vrouwen langer te vervolgen. Dit heeft geresulteerd in de prospectieve multicentrum studie die beschreven staat in **Hoofdstuk 11**. Vrouwen met type 1 of type 2 diabetes met asymptomatische bacteriurie op baseline werden vergeleken met vrouwen met diabetes zonder asymptomatische bacteriurie met betrekking tot de nierfunctie en het ontwikkelen van hypertensie. In totaal werden 644 vrouwen geïncludeerd (296 met type 1 diabetes en 348 met type 2 diabetes), van wie 110 vrouwen (17%) asymptomatische bacteriurie hadden aan het beginpunt. De gemiddeld follow-up duur was 6 jaar. Bij vrouwen zonder asymptomatische bacteriurie daalde de kreatinineklaring (maat voor de nierfunctie) gemiddeld van 97 naar 88 ml/minuut en bij vrouwen met asymptomatische bacteriurie van 87 naar 76 ml per minuut. In de multivariate analyse, waarbij gecorrigeerd werd voor leeftijd, follow-up duur, duur van de diabetes en microalbuminurie (eiwit in de urine), kon geen verschil worden aangetoond in de relatieve of absolute vermindering van de kreatinineklaring, ook niet als vrouwen met type 1 en type 2 diabetes apart werden geanalyseerd. Hoewel vrouwen met asymptomatische bacteriurie vaker hypertensie ontwikkelden,

verdween dit verschil na correctie voor potentieel verstorende factoren.
Concluderend leidt het hebben van asymptomatische bacteriurie niet tot een snellere achteruitgang van de nierfunctie of tot eindstadium nierfalen bij vrouwen met of zonder diabetes mellitus. Hoewel vrouwen met diabetes mellitus en asymptomatische bacteriurie geen hoger risico lopen op het ontwikkelen van hypertensie, werd er wel een sterke correlatie aangetoond tussen asymptomatische bacteriurie en het ontwikkelen van hypertensie bij de gezonde vrouwelijke populatie.

Tot slot worden de resultaten van de verschillende hoofdstukken nogmaals samengevat in **Hoofdstuk 12**, waarbij de bevindingen worden bediscussieerd in het licht van al bestaande data uit de literatuur. Een belangrijk doel van onze studies was het onderzoeken of asymptomatische bacteriurie bij vrouwen met diabetes op de lange termijn kan leiden tot schadelijke gevolgen en vooral of deze populatie die toch al at risk is voor het krijgen van nierfunctieverslechtering een nog hoger risico loopt door het hebben van bacteriën in de urine. Dit hebben wij niet aan kunnen tonen na een langdurige follow-up van zes jaar. Het lijkt daarom gerechtvaardigd om te concluderen dat asymptomatische bacteriurie in deze patiëntengroep niet schadelijk is en ook niet opgespoord en behandeld hoeft te worden.

Twelve

Het leukste van het schrijven van een dankwoord is dat er allerlei herinneringen boven komen uit de afgelopen onderzoeksjaren. Zonder die leuke momenten en de steun van velen zou dit proefschrift nooit afgekomen zijn. Een paar mensen wil ik in het bijzonder bedanken.

Ten eerste wil ik alle patiënten bedanken die hebben deelgenomen aan de verschillende onderzoeken die in dit proefschrift staan beschreven. Zonder u is er geen onderzoek mogelijk. Hopelijk dragen de resultaten van dit proefschrift een klein steentje bij aan de inzichten over uw ziekte.

Geachte promotor Professor Dr. I.M. Hoepelman, beste Andy, door jouw enthousiasme en drijfkracht ben ik aan dit onderzoek begonnen en mede daardoor heb ik het ook afgerond. Je moet het toch als een kunst beschouwen dat iemand een ander kan inspireren om jarenlang een asymptomatische aandoening te bestuderen. Bedankt dat je mij de gelegenheid hebt geboden het op mijn eigen manier te doen en me ook de ruimte gaf het onderzoek af te wisselen met de kliniek.

Geachte co-promotor Dr. S.E. Geerlings, beste Suzanne, voor mij ben jij de ideale combinatie van collega, baas en vriendin. Ook nadat je naar Amsterdam bent gegaan bleef je enthousiast en betrokken, hartelijk bedankt daarvoor. Kraamvisites kunnen prima gecombineerd worden met werkbesprekingen. Ik hoop dat we contact houden.

Geachte promotor Professor Dr. R.P. Stolk, beste Ronald, bedankt voor de frisse wind vanuit de andere hoek. Dankzij jou kon ik klinische data omzetten in epidemiologisch verantwoorde resultaten. Gelukkig hebben ze in Groningen ook de e-mail ontdekt. Veel geluk in het noorden.

De leden van de beoordelingscommissie, Prof. dr. D.E. Grobbee, Prof. dr. J.L.H.R. Bosch, Prof. dr. G.E.H.M. Rutten en Prof. dr. P. Speelman, u wil ik danken voor uw tijd en aandacht.

Heel veel dank gaat uit naar Ellen Brouwer, analiste. Beste Ellen, van jou heb ik veel geleerd. Van het hanteren van een pipet tot het bewaren van mijn geduld als de FACS-resultaten weer eens anders uitpakten dan gehoopt. Bedankt voor al het werk dat jij verzet hebt, voor je zorgvuldigheid die ervoor zorgde dat we die honderden potjes urine uit elkaar konden houden en vooral ook voor je gezelligheid. Door jou weet ik de geur van *Escherichia coli* te waarderen.

Dr. F.E.J. Coenjaerts, beste Frank, dankjewel voor de keren dat jij op je bescheiden manier een discussie in goede banen hebt weten te leiden en ons met praktische tips weer op weg hebt geholpen. Veel succes met je eigen onderzoek.

De medewerkers van het Eijkman Winkler Instituut voor Medische Microbiologie wil ik bedanken voor het met raad en daad te hulp schieten van weer zo'n dokter die zo nodig labwerk wilde doen waar zij niet voor opgeleid was.

De internisten van de polikliniek diabetologie van het UMC Utrecht en dr. Paetrick Netten van het Jeroen Bosch Ziekenhuis ('s Hertogenbosch) wil ik bedanken voor hun hulp bij het includeren van de diabetespatiënten. De microbiologen en analisten van het laboratorium voor Medische Microbiologie van het UMC Utrecht en dr. Peter Schneeberger en de analisten van het laboratorium voor Medische Microbiologie van het Jeroen Bosch Ziekenhuis, voor het kweken van de urine en het bewaren van de stammen.

Professor Dr. D.E. Grobbee en Dr. P.H.M. Peeters, bedankt voor het mogelijk maken en het kritisch beoordelen en becommentariëren van de studies met het DOM-cohort (hoofdstuk 8 en 9). Professor Dr. A.W. Hoes, bedankt voor de kritische noten bij hoofdstuk 6. Bernard Slotboom wil ik bedanken voor zijn hulp bij de databases van het DOM-cohort. Dr. A.J. de Neeling bedank ik voor het ter beschikking stellen van de resistentie cijfers van regio Utrecht die we nodig hadden voor de controlegroep in hoofdstuk 5. Dr. F.Th. de Charro, voor het mogelijk maken dat de gegevens van het DOM-cohort werden gekoppeld aan het fantastische registratiesysteem van Stichting RENINE.

Mede-onderzoekers van de vrouwenvleugel, lieve Irene, Marianne, Mirelle, Stefan, Fieke, Ilja, Saskia en Jan-Jelrik. Alle onderzoeksfrustraties kun je relativeren tijdens een kop koffie met de meesterlijk geklopte melk van Irene. Bedankt voor de lunches, de koffie-uurtjes, de liters urine die jullie ter beschikking stelden als ik controlepersonen nodig had en niet te vergeten voor het helpen zoeken naar 30 jaar oude potjes urine in een levensgrote vriezer bij min twintig graden Celsius.

Mijn collega's uit het St. Antonius Ziekenhuis, beste MDL-artsen, internisten, arts-assistenten en medewerkers van de afdeling MDL-ziekten, hartelijk bedankt voor het creëren van een leuke en inspirerende werkomgeving en het duidelijk maken dat er nog meer onderwerpen bestaan buiten urine…

Mijn paranimfen, lieve Anouk en Brenda, we hebben een hoop meegemaakt samen, ik ben blij dat jullie me ook nu weer terzijde willen staan. Mijn andere vrienden, jullie betekenen veel voor mij om dingen die niets met dit proefschrift te maken hebben. Het wordt tijd voor leuke dingen.

Sommige dingen hoef je niet uit te spreken, andere mogen best eens zwart op wit gedrukt worden. Mijn ouders en mijn zus wil ik bedanken voor alle liefde en steun die ze mij geven. Pap, ik denk dat jij de enige niet-medicus zal zijn die dit boekje (bijna) helemaal uit zal proberen te lezen.

Eric, mijn liefje, bedankt voor heel veel meer dan ik hier op wil noemen. Jij maakt mij gelukkig en daar kan niets tegenop. Pepijntje, ik ben blij dat jij er bent.

…dus zo voelt het als je een Oscar wint.

Twelve

Summary and
General Discussion
Samenvatting
Dankwoord
Curriculum Vitae

Ruby Meiland, dochter van Jan Meiland en Lenneke Meiland-Smit, werd geboren op 7 januari 1973 in Zaandam. Van 1985 tot 1991 volgde zij de middelbare schoolopleiding aan het Theresialyceum in Tilburg. Zij begon in 1991 met de studie Geneeskunde aan de Universiteit van Amsterdam. Van augustus tot december 1994 verbleef zij in Kaoma District, Zambia, waar zij epidemiologisch onderzoek deed naar *Plasmodium falciparum* bij schoolgaande kinderen. Op 31 mei 1996 behaalde zij het doctoraalexamen Geneeskunde en op 25 november 1998 het artsexamen. Hierna is zij van december 1998 tot juni 2000 werkzaam geweest als AGNIO Interne Geneeskunde in Ziekenhuis Hilversum (Dr. S. Lobatto). In juni 2000 is zij gestart met het onderzoek beschreven in dit proefschrift in het Universitair Medisch Centrum (UMC) Utrecht (promotor Prof. dr. I.M. Hoepelman). Van 1 september 2002 tot 1 september 2004 volgde zij de (voor-)opleiding Interne Geneeskunde in het Sint Antonius Ziekenhuis Nieuwegein (opleider Dr. H.C.M. Haanen). Vanaf mei 2005 is zij gestart met de opleiding tot maag-, darm- en leverarts in het Sint Antonius Ziekenhuis Nieuwegein (opleiders Dr. R. Timmer en Prof. dr. M. Samsom). Op 10 augustus 2002 is zij getrouwd met Eric van Dal. Zij hebben een zoon Pepijn.

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List of Abbreviations

AFU	arbitrary fluorescence units
ASB	asymptomatic bacteriuria
AUC	area under the (receiver operation characteristic) curve
BSA	bovine serum albumin
CFU	colony forming unit
CI	confidence interval
CT	computed tomography
DM	diabetes mellitus
DOM	Diagnostisch Onderzoek Mammacarcinoom
DMEM	Dulbecco's Modified Eagle Medium
EPIC	European Prospective Investigation into Cancer and Nutrition
FACS	Fluorescence Activated Cell Sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFR	glomerular filtration rate
GHb	glycosylated hemoglobin
H	hour
HbA1c	hemoglobin A1c
HPF	high power field
IDSA	Infectious Diseases Society of America
IMDM	Isocoves Modified Dulbecco's Medium
LB	Luria broth
MIC	Minimal Inhibitory Concentration
MRHA	mannose-resistant hemagglutination
MSHA	mannose-sensitive hemagglutination
NCCLS	National Committee for Clinical Laboratory Standards
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RENINE	Registratie Nierfunctievervangende therapie Nederland
ROC	receiver operating characteristic
SD	standard deviation
SE	standard error of the mean
SPSS	Statistical Product and Service Solutions
RPM	rounds per minute
THP	Tamm Horsfall Protein
TMP-SMX	trimethoprim-sulfamethoxazole
UNG	uracil-N-glycosylase
UTI	urinary tract infection
WBC	white blood cells