Antimicrobial Use and Antimicrobial Resistance in Community-Acquired Urinary Tract Infections

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Antibiotica gebruik en antibioticaresistentie bij urineweginfecties in de eerstelijnsgezondheidszorg

Thesis

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MARLIES MULDER

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Erasmus University Rotterdam

Promotors:	Em.prof.dr. B. H. Ch. Stricker
	Prof.dr. A. Verbon
Other members:	Prof.dr. P. J. E. Bindels
	Prof.dr. S. J. de Vlas
	Prof.dr. S. E. Geerlings

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Chapter 1

General Introduction and Outline of this Thesis

Infectious diseases are among the diseases earliest recognized by mankind.[1] The World Health Organization (WHO) defines them as:

"*Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; the disease can be spread directly or indirectly, from one person to another.*"[2]

The scale on which infectious diseases have spread among humans in history has substantially increased with the growing population, especially by the shift to agrarian communities and further civilization, followed by widespread trade and travel. These factors have created an increase in human-human and animal-human interactions.[1] However, the death rate from infectious diseases has decreased in the latest centuries due to hygiene and healthcare improvements. Moreover, new therapies emerged, such as penicillin, discovered in 1928 by Alexander Fleming and sulphonamides, discovered in 1932 by Domagk. Both drugs have been effectively used to treat a variety of bacterial infections.

Antimicrobial resistance

Although many antimicrobial drugs have been developed since penicillin, resistance of pathogens to most antimicrobial drugs have already occurred. Antimicrobial resistance (AMR) is defined by the WHO as:

"*the ability of a microorganism (like bacteria, viruses and other parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it*".[3]

Alexander Fleming already warned in an interview in The New York Times in 1945, shortly after winning the Nobel Prize of Medicine, that misuse of penicillin could result in selection of resistant bacteria. He said:

"The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism."

Indeed, in the second half of the twentieth century, AMR became an increasing problem for modern medicine. In the last decades, resistance to common bacteria has reached alarming levels and the WHO described AMR as a threat to global public health in a worldwide report published in 2014. The organization warned that AMR may result in a post-antibiotic era, in which common infections cannot be treated anymore and are thus potentially lethal.[4]

Several other reports have already shown the consequences of AMR in the last decade. The Centre for Disease Control and Prevention (CDC) estimated in a report in 2013 that more than two million individuals suffer from antibiotic-resistant infections every year with a mortality of

23,000 patients.[5] A joint report of the European Centre for Disease Prevention and Control (ECDC) and the European Medicine Agency (EMA) from 2009 estimated that approximately 25,000 inhabitants of the EU die each year because of an infection with multidrug-resistant bacteria and that infections due to these bacteria result in extra healthcare costs and productivity losses of at least 1.5 billion euros each year.[6] Also, the ECDC published a report with data from the European Antimicrobial Resistance Surveillance Network, estimating that approximately 670,000 infections with resistant bacteria have occurred in 2015 resulting in over 33,000 attributable deaths in the EU and EEA.[7] According to a report from Lord Jim O'Neill, commissioned by the United Kingdom government, this trend will continue with as a result that AMR in 2050 will catch-up cancer as cause of death resulting in 10 million deaths a year, when nothing changes.[8] On the other hand, a later study showed that the data that we have at this moment are probably too unreliable to make such hard statements.[9]

New antimicrobial drugs and good antimicrobial stewardship

One of the possibilities to combat infections caused by resistant bacteria, is the development of new antimicrobial drugs. However, fourteen new classes of antimicrobial drugs have been introduced between 1935 and 2003, but only one antimicrobial drug (mainly from existing classes) was approved per year from 2004 to 2009. Since 2014, this situation slowly improved, but the number of recently approved antimicrobial drugs remains low.[10, 11] This may have been expected, since the development of antibiotic drugs is risky and less profitable than the development of drugs for chronic diseases.[10] Therefore, we should be careful with the antimicrobial drugs that we have. A promising approach are good antimicrobial stewardship programs, defined by the WHO as interventions designed to promote the optimal use of antimicrobial agents. These interventions include, the correct indication for use, drug choice, dosing, route, and duration of administration. The goal of these programs is 3-fold. First, these programs are meant to help healthcare workers to treat each patient with the most appropriate antimicrobial, using the four D's of optimal antimicrobial therapy: right Drug, right Dose, De-escalation of therapy and right Duration. Second, these programs aim to prevent misuse, overuse or abuse of antimicrobial therapy. Third, their goal is to minimize the development of antimicrobial resistance by assessing risk factors for developing AMR.[10]

Factors that play a role in antimicrobial resistance

Antimicrobial drug use

The most well-known risk factor for AMR is the use of antimicrobial drugs. This was shown in a large systematic review with 24 studies, including 14,238 participants with urinary tract

infections (UTIs) and 2605 participants with bacterial respiratory tract infections. The study showed that patients using antimicrobial drugs are at risk to have infections with or become carriers of bacteria resistant to the antimicrobial drug they used. AMR was most frequent in the first month after treatment and increased with longer duration of antimicrobial therapy and number of treatments.[12]

Natural sources

It is a misconception to assume that antimicrobial resistance genes have emerged only after the introduction of antimicrobial drugs. For example, over 150 naturally occurring antimicrobial resistance genes have been found in remote Antarctic surface soils within the undisturbed Mackay Glacier region.[13] Also, analyses of ancient DNA from 30,000-year-old Beringian permafrost sediments showed genes encoding resistance to beta-lactams, tetracyclines, and glycopeptides. Interestingly, the structure and function of the VanA vancomycin resistance element showed similarities with modern variants.[14] Moreover, a study that investigated the gut microbiota of (pre-)Inca (10th – 14th century) and Italian nobility mummies (15th and 16th century) showed the presence of resistance genes. The Inca mummies had a higher proportion of DNA segments (3.29%) that were associated with resistance genes than the Italian mummies (1.58%), but both were lower than in modern Amazonian (4.96%) and modern European populations (3.65%). The sequenced elements were associated with resistance to vancomycin and multi-drug transporters, but also with fosfomycin, chloramphenicol, aminoglycoside, macrolide, sulfa, quinolone and tetracycline resistance.[15] Furthermore, the first beta-lactamase was already described in 1940, which was before antimicrobial drugs were released for use in medical practice.[16] However, antimicrobial resistance often comes at the cost of reduced fitness of the micro-organism. Therefore, selective pressure, that is during use of antimicrobial drugs, is important for spread of AMR.[17]

International travel

Travel is a risk factor for the spread of infectious diseases, but also for the spread of AMR. Individuals who travel to areas with high AMR prevalence are likely to return colonized by bacteria resistant to antimicrobial drugs.[18, 19] In a study with over 2000 Dutch travelers, more than one-third of the individuals that were extended-spectrum beta-lactamase (ESBL) negative before travel acquired an ESBL during international travel, which persisted up to 12 months after return.[20]

Animals

Horizontal gene transfer (HGT) is the movement of genetic information between two organisms, by which bacteria can exchange genes, such as antimicrobial resistance genes.[21] Although still under debate, HGT is often called in one breath with the possible transfer of AMR from animals to humans. Antimicrobial drugs are often prescribed, in the pig, cattle and chicken industries, and also in the farmed seafood industry.[22] A recent study even estimated a global increase of

use of antimicrobials in food animals by two thirds in the period 2010-2030.[23] Indeed, animals have been shown to be an important reservoir of antimicrobial resistance genes,[24] which may be transferred to humans either via direct contact or via ingestion of food, such as meat or crops contaminated with water or soil. [25] For example, soil treated with manure is full of antimicrobial resistance genes and can contaminate groundwater or streams, used to irrigate vegetables and fruits.[26] Other studies have suggested possible transfer of resistant bacteria via meat consumption.[27] Moreover, similarities have been suggested between ESBL-genes from *E.coli* in broilers, retail chicken and clinical isolates from humans.[28-30] However, these data could not be confirmed in more recent studies.[31] To prevent the widespread resistance to antimicrobial drugs that are critical for human health, the WHO has established a list of critically important antimicrobials. This list uses several criteria to distinguish between "critically important", "highly important" and "important" antimicrobial drugs for human health and it includes amongst others 3rd and higher generations of cephalosporins, glycopeptides, macrolides and quinolones as drugs with highest priority.[32]

One Health is the collaboration between multiple scientific disciplines with as goal to improve the health of people, animals and the environment, recognizing that these are connected. It has led to a worldwide strategy stimulating interdisciplinary collaborations between all aspects of health care for humans, animals and the environment, including AMR.[33]

Gut microbiota

One of the locations that is hypothesized to facilitate HGT is the gut microbiota. This is the community of microbes living in the gut, which is quite unique for every individual and could therefore be seen as the fingerprint of the gut.[34] In the last decades, the interest in the different human microbiota has grown, and especially the gut microbiota has been associated with many diseases, varying from inflammatory bowel diseases and gastrointestinal malignancies to metabolic disorders, such as diabetes, but also allergies, autism, psychiatric and neurologic disorders.[35] The gut microbiota is a complex entity that can be described in many ways. Commonly used is the diversity of the microbiota. The alpha-diversity, for example calculated according to Shannon, is the diversity of microbes within a community, taking into consideration both the number of different species (richness) and the proportion of the species. A community will have a high alpha-diversity when there are many different microbes with similar abundances. The beta-diversity was originally described by Whittaker in 1972 as "*the extent of species replacement or biotic change along environmental gradients*".[36] It is a measure of dissimilarity between the compositions of microbiota of two different samples (e.g. from 2 different individuals) and is often calculated according to the method of Bray-Curtis.

Recent techniques also allow us to study the diversity and dynamics of the antibiotic resistance genes harboured by the gut. The total number of resistance genes in the gut is called the gut resistome. Well-known resistance genes and possibly the ones we fear the most are the beta-

lactamases (BLs) and especially the ESBLs. Bacteria that carry these genes are able to continue to grow in the presence of many beta-lactam antimicrobial drugs and infections caused by these bacteria are therefore difficult to treat.

Urinary tract infections

Urinary tract infections (UTIs) are among the most common bacterial infections and account for an important share of antimicrobial drug use. There is a large difference in epidemiology of UTIs between men and women. In men, UTIs are most common in the higher age groups.[37] Although, UTIs in men are often associated with different and more complex pathology, in the last decade, uncomplicated UTIs, such as a cystitis has increasingly acknowledged.[38-40] UTIs are less common in men than in women with 258 episodes per 10.000 men versus 1656 episodes per 10.000 women in 2014.[37] In women, UTIs were the most common reason to consult a general practitioner (GP) in 2015.[41] UTIs in women are common in all age groups, with peaks in young adulthood and after menopause.[37] In 2014, in girls of 10-14 years old 408 episodes of UTIs were described per 10.000 women, compared to 1471 episodes in 15-19 years old girls and 1793 episodes per 10.000 in 20-24 years old women. After menopause, the number of episodes is even higher, 1759 episodes per 10.000 women in the age group of 60-64 to 2653 in 70-74, 3766 in 80-84 and even 5857 episodes per 10.000 women in women of 85 years and older.[37] Most of these UTIs are cystitis but in some cases, the bacteria spread to the renal pelvis causing pyelonephritis. A pyelonephritis can evolve into a urosepsis, when the infection spreads into the blood stream. Urosepsis nowadays still has a high mortality rate.[42]

Recurrent UTIs, defined as ≥2 UTIs in the last six months or ≥3 UTIs in the last year occur often, even in young healthy women. In a study among 113 college women (mean age 21.3 years) with a UTI, 27% experienced at least one recurrence within 6 months.[43] In a Finnish study, with 179 women (17-82 years), 44% had a least one recurrence within a year.[44] In a large case-control study among women with and without recurrent UTIs, sexual intercourse was the strongest risk factor for recurrent UTIs, followed by age at first UTI; UTI history in mother, use of spermicides and a new sex partner during the last year.[45] Recurrent UTIs seem “to run into the family”, as shown in a case-control study, including 1,261 women of 18-49 years old.[46] It has still not been elucidated whether recurrent UTIs are re-infections with a new pathogen invading the urogenital system or recurrences of a not adequately eliminated pathogen.[47]

UTIs are often caused by gram-negative organisms, such as *Escherichia coli (E.coli),* which is the causative pathogen in 70-80% of community-acquired UTIs. In the Netherlands, treatment of UTIs is started by GPs according to the Dutch GP guideline. This guideline leave room for a wait-and-see policy in the treatment of uncomplicated UTIs in healthy, non-pregnant women.

Nevertheless, many episodes of UTIs are treated with antimicrobial drugs. The Dutch guideline has been changed several times during the last three decades and is adjusted according to the current resistance rates. Nowadays, nitrofurantoin has replaced trimethoprim as first-choice in the treatment of uncomplicated infections in women, whereas ciprofloxacin is the recommended first-choice for complicated infections.[38, 39, 48]

Antimicrobial resistance in urinary tract infections

The recommendations in the guideline are determined by using the most recent AMR rates. Although, the prevalence of AMR in uropathogens in The Netherlands is quite stable for the past few years,[49] AMR rates in uropathogens in some parts of the world are rapidly increasing. A systematic review, including 54 studies showed increasing trends in ciprofloxacin resistance in both community- and hospital-acquired UTIs caused by *E.coli* between 2004 and 2014.[50] Furthermore, a study in 12 countries in Europe (Belgium, Bulgaria, the Czech Republic, France, Germany, Greece, Ireland, Italy, Norway, Portugal, Slovenia and Sweden) showed an increase from 9.6% to 12.0% of ESBL-*E.coli* between 2011 and 2014 in the overall EU/EEA population.[51] Resistance rates in some countries are alarming. For example, 34.4% *E.coli's* from a hospital in India in 2004/2005 were ESBL-producing and 69% were ciprofloxacin resistant.[52] In the Netherlands, the prevalence of ESBL-*E.coli* is lower, varying from 3% in GP patients to 7% in ICU patients in 2018.[49]

Aim of the thesis

Because of the increasing AMR rates, common infections, such as UTIs are more difficult to treat. This leads to increased morbidity and mortality and the risk that these very common infections cannot be treated with antimicrobial drugs in the future. To reduce this risk, more knowledge is needed about risk factors for AMR in UTIs. The aim of this thesis is to study antimicrobial drug use in UTIs, risk factors for AMR in UTIs and the role of the gut and genitourinary tract microbiota.

Primary research questions

1. Can good antibiotic stewardship be evaluated using a real-life primary care database?
2. What are risk factors for antimicrobial resistance in UTIs in a middle-aged and elderly population?
3. What is the effect of antimicrobial drug use on the gut and urinary tract microbiota?
4. Does the composition of the gut microbiota play a role in the development of UTIs.

Outline of this thesis

In **Chapter 2**, the large IPCI database was used to describe trends of antimicrobial drugs prescribing for UTIs by GPs in the Netherlands in the period 1995-2014. Subsequently, these prescribing trends were compared with the recommendations according to the Dutch GP guideline to assess antibiotic stewardship.

In **Chapter 3,** risk factors for AMR in UTIs were determined, including risk factors for ciprofloxacin resistance. Furthermore, the associations between the use of different antimicrobial drug groups and trimethoprim resistance in UTIs and the effects of diet on antimicrobial resistance for several antimicrobial drugs were investigated.

In **Chapter 4,** the (long-term) effects of antimicrobial drugs on both the genitourinary tract microbiota and the gut microbiota were described. Additionally, the epidemiology of the resistance genes TEM, SHV, CMY and CTX-M in the gut in a middle-aged and elderly population and possible risk factors for being a carrier of these genes were studied. Finally, the role of the microbiota in the development of UTIs in women without a recent UTI history was investigated.

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Chapter 2

Antimicrobial Drug Prescribing in Urinary Tract Infections

Chapter 2.1

Trends of Prescribing Antimicrobial Drugs for Urinary Tract Infections in Primary Care in the Netherlands: a Population-Based Cohort Study.

Marlies Mulder, Esmé Baan, Annelies Verbon, Bruno Stricker, Katia Verhamme

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Abstract

Objective Urinary tract infections (UTIs) are an important reason to consult a general practitioner (GP). Here, we describe antimicrobial drug prescribing patterns for UTIs by GPs in relation to the Dutch primary care guidelines.

Methods We conducted a population-based cohort study in the Dutch Integrated Primary Care Information (IPCI) database, which encompasses approximately 2.5 million patients. All patients aged ≥12 years with at least 1 year of follow-up from 1996 to 2014 were extracted from the database. The number of prescriptions and choice of drug type were investigated over time and in different age categories. The choice of antimicrobial drug classes for UTIs and the duration of nitrofurantoin use in women were compared with the Dutch primary care guidelines of 1989, 1999, 2005 and 2013.

Results The source population comprised 1,755,085 patients who received 2,019,335 antimicrobial drug prescriptions; 401,655 (35.1%) prescriptions were for UTIs (45.2% in women and 12.6% in men). The proportion of prescriptions for UTIs within all prescriptions with an indication code increased from 5.2% in 1996 to 14% in 2014 in men and from 28% in 1996 to 50% in 2014 in women. In men, UTIs were most frequently treated with fluoroquinolones during the entire study period, whereas fluoroquinolones were only advised as first choice in the latest guideline of 2013. In women, UTIs were increasingly ($p<0.05$) treated with nitrofuran derivatives with a statistically significant difference after implementation of the guideline of 2005. Compliance to the advised duration of nitrofurantoin prescriptions in women has increased since the guideline of 2005.

Conclusions Antimicrobial drug prescribing for UTIs seemed to have increased over time. Prescribing in line with the UTI guidelines increased with regard to choice and duration of antimicrobial drugs. We showed that databases like IPCI, in which prescription and indication are monitored, can be valuable antibiotic stewardship tools.

Introduction

Urinary tract infections (UTIs) are among the most common infections in humans. UTIs cause a substantial burden of disease with major economic consequences.[1 2] In women, UTIs are the most common reason to consult a general practitioner (GP) in The Netherlands with 232 contacts per 1000 women in 2014. In men, this number was substantially lower: 37 contacts per 1000 men.[3]

In most cases UTIs are treated with antimicrobial drugs. The choice of antimicrobial drug depends on the antimicrobial sensitivity of pathogens in urinary cultures (if taken), severity of the symptoms and potential comorbidities of the individual. Based on the resistance patterns of *Escherichia coli* in The Netherlands, Dutch guidelines on the treatment of UTIs have changed several times in the last few decades. The latest Dutch guideline for the treatment of UTIs in primary care was released in 2013 and recommends nitrofurantoin as first choice in all patients with cystitis, including men. In case of signs of tissue invasion, ciprofloxacin is recommended as first choice.[4] Additionally, the recommended duration of treatment has changed. The recommended duration of treatment with nitrofurantoin for cystitis in women was 3 days in the guidelines of 1989 and 1999, whereas it has been extended to 5 days in the guidelines of 2005 and 2013 (**Table 1**).[4–7]

Previous research shows that the use of antimicrobial drugs in the Netherlands in general has increased since 2005 until approximately 2012, most prominently in elderly patients, but has decreased again since then.[8 9] Since UTIs are a major reason to consult a GP, the use of antimicrobial drugs for UTIs contributes significantly to total antimicrobial consumption. Therefore, antibiotic stewardship focusing on (inappropriate) antimicrobial drug prescribing for UTIs is an important target in the fight against antimicrobial resistance (AMR). This study aims to study the choice of antimicrobial drugs prescribed for UTIs by GPs and the duration of nitrofurantoin use over time in relation to the Dutch national guidelines (of 1989, 1999, 2005 and 2013) for the treatment of UTIs in primary care using a large electronic primary care database.

Materials and methods

Data source

This study was conducted using data from the Integrated Primary Care Information (IPCI) database, which is a longitudinal observational dynamic database containing the records from more than 450 general practices throughout the Netherlands.[10] Briefly, IPCI contains the complete electronic medical records of ~2 500 000 patients, composed of, among others, data

Table 1: Overview of the treatment of UTIs according to the Dutch guidelines for GPs

	1989	1999	2005	2013
Women				
Cystitis	Trimethoprim (J01EA) *or* Sulfamethizol (J01EB) *or* Nitrofurantoin (J01XE) ***3 days***	Nitrofurantoin (J01XE) ***3 days*** *or* Trimethoprim (J01EA)	*1st choice*: Nitrofurantoin (J01XE) ***5 days*** *2nd choice*: Trimethoprim (J01EA) *3rd choice*: Fosfomycin (J01XX)	*1st choice*: Nitrofurantoin (J01XE) ***5 days*** *2nd choice*: Fosfomycin (J01XX) *3rd choice*: Trimethoprim (J01EA)
UTI with tissue invasion	Amoxicillin (J01CA)	*1st choice*: Amoxicillin-clavulanic acid (J01CR) *2nd choice*: Sulfamethoxazole-trimethoprim (J01EE)	*1st choice*: Amoxicillin-clavulanic acid (J01CR) *2nd choice*: Sulfamethoxazole-trimethoprim (J01EE) *or* Fluoroquinolone (J01MA)	*1st choice*: Ciprofloxacin (J01MA) *2nd choice*: Amoxicillin-clavulanic acid (J01CR) *3rd choice*: Sulfamethoxazole-trimethoprim (J01EE)
Men				
Cystitis	UTI in men should always be considered to be prostatitis	Nitrofurantoin (J01XE) *or* Trimethoprim (J01EA)	*1st choice*: Nitrofurantoin (J01XE) *2nd choice*: Trimethoprim (J01EA)	*1st choice:* Nitrofurantoin (J01XE) *2nd choice*: Trimethoprim (J01EA)
UTI with tissue invasion	Trimethoprim (J01EA) *or* Amoxicillin (J01CA)	*1st choice*: Amoxicillin-clavulanic acid (J01CR) *2nd choice*: sulfamethoxazole-trimethoprim (J01EE)	*1st choice*: Amoxicillin-clavulanic acid (J01CR) *2nd choice*: Sulfamethoxazole-trimethoprim (J01EE) *or* Fluorochinolone (J01MA)	*1st choice*: Ciprofloxacin (J01MA) *2nd choice*: Amoxicillin-clavulanic acid (J01CR) *3rd choice*: Sulfamethoxazole-trimethoprim (J01EE)

Please note that information from guidelines on the treatment of pregnant women and also the treatment of risk groups such as patients with diabetes or abnormalities of the urinary tract, differs and is not described. Furthermore, the treatment duration of men is in all case longer than that in women.

on diagnoses (coded and free text) and prescriptions coded according to the Anatomical Therapeutical Chemical (ATC) classification of the WHO.[11] The system complies with European Union guidelines on the use of data for medical research and has been proven valid for pharmacoepidemiological studies. More detailed information on IPCI has been described elsewhere.[10]

Study population

The study cohort comprised patients aged ≥12 years with at least 1 year of valid database history in the IPCI database. The study period was from 1 January 1996 until 1 January 2015. Follow-up started from the following, whichever occurred last: start of the study period, age of 12 years or reaching a minimum of 12 months of database history. Follow-up ended when a patient left the database or died or at the end of the study period, whichever occurred first. Gender, age at the time of prescriptions)and follow-up time (time of each patient in the database) were assessed for all patients.

Prescriptions

From the database, we selected all prescriptions for antimicrobial drugs prescribed during the study period, using an automatic search on the ATC code 'J01', which is the ATC code for antimicrobial drugs. All prescriptions of antimicrobial drugs were further categorized by ATC drug class, for example, J01AA (tetracyclines) (supplementary table s1). We analyzed all prescriptions of ATC class J01CA (penicillins with extended spectrum), J01CR (combinations of penicillins, including beta-lactamase inhibitors), J01EA (trimethoprim and derivatives), J01EE (combinations of sulfonamides and trimethoprim, including derivatives), J01MA (fluoroquinolones), J01XE (nitrofuran derivatives) and J01XX (other antimicrobials [mainly fosfomycin]). In addition, the duration of all prescriptions of nitrofurantoin (J01XE01) (which in the Netherlands is only prescribed for UTIs) was assessed.

Indication of use of antimicrobial drugs

Indication of use was assessed through an automatic search on disease-specific codes. Antimicrobial drug prescriptions were linked to the indication of use through a unique patient identifier linking a prescription to a diagnosis using the International Classification of Primary Care (ICPC)-1 codes (version 5). These ICPC codes were categorized in the following indications: UTIs, skin infections, respiratory infections, ear infections or other infections. All prescriptions without an ICPC code were assigned to the group: 'no code for indication of use'. Urethritis was included in the group of 'other' infections and not in the UTI group because of the distinct pathophysiology.

Analyses

The total number of prescriptions for UTIs, 'other infections' (including respiratory infections, skin infections and ear infections) and prescriptions without an indication code were calculated

for the complete study time and per year. Additionally, the proportion of UTI prescriptions per calendar year was calculated with all antimicrobial drug prescriptions with an indication code as denominator. Also, all nitrofurantoin prescriptions were analyzed per year.

Since both the number of users and the total number of antimicrobial drug prescriptions are interesting with regard to the study of AMR, we studied both. Because of the dynamic nature of the study cohort, the annual frequency of antimicrobial drug prescriptions was calculated by dividing the total number of antimicrobial drug prescriptions by the total number of person-years (PYs). The annual number of users per calendar year was calculated by dividing all users by the total number of PY in that specific year. With regard to the calculation of users, if an individual received prescriptions of more than one antimicrobial drug class in 1 year, the individual contributed data to the different classes. However, if an individual received two or more prescriptions of the same drug in 1 year, the individual contributed only once as a user of this specific drug class. The frequency of prescriptions and users were studied by age (12–17, 18–25, 26–35, 36–45, 46–55, 56–65, 66–75, 76–85 and ≥86 years age categories), gender and calendar year. The prescribing of antimicrobial drugs by GPs was compared with the recommendations according to the national guidelines (table 1).[4–7]

We intended to investigate if improved coding over time has influenced the results of the frequency of prescribing antimicrobial drug prescriptions for UTIs in general, independent of the drug class. Therefore, we also studied the frequency of nitrofurantoin prescriptions over time (since nitrofurantoin was only prescribed for UTIs), and we studied the proportion of prescriptions for UTIs within total prescriptions with an indication code (thus including all antimicrobial drug prescriptions with information on indication of use).

Furthermore, all nitrofurantoin prescriptions prescribed to women during the study period were selected, and duration of use was categorized in 3, 5, 7 days or in 'other' (when the number of days of use was unknown or different from 3, 5, or 7 days). The proportion of each category with the total number of prescriptions of nitrofurantoin in women as denominator was calculated by year.

Finally, we used a time series ARIMA model (in SPSS V.24) in order to determine the effect of the implementation of the guideline of 2005.[12] The calendar year was first univariably added to the analysis to study differences of antimicrobial drug prescribing over time. Next, we ran a model including calendar year and the intervention (implementation of the guideline). When the p value of the interaction term was <0.05, the difference in slope before and after the implementation of the guideline was significant, implying a significant effect of the implementation of this guideline.

Results

The study population comprised 1,755,085 patients aged ≥12 years with a mean follow-up time of 3.31 years. A total of 671,251 (38.2%) patients were prescribed at least one antimicrobial drug during the study period: 271,772 (40.5%) men and 399,479 (59.5%) women. In total, they received 2,019,335 antimicrobial drug prescriptions (mean of 3 prescriptions per person) during the study period. Of these prescriptions, 1,144,810 (56.7%) could be linked to an indication, namely, 528,464 (46.2%) antimicrobial drug prescriptions for respiratory infections; 401,655 (35.1%) for UTIs; 157,900 (13.8%) for skin infections; 29,984 (2.6%) for ear infections and 26,807 (2.3%) for 'other' infections.

Antimicrobial drug prescriptions for UTIs by calendar year

For all years combined and only considering those prescriptions with indication of use, prescriptions for UTIs were 12.6% of the total prescriptions in men and 45.2% in women. The total number of prescriptions per year for UTIs increased from 9 (men) and 88 (women) prescriptions per 1000 PY in 1996 to 19 and 153 prescriptions per 1000 PY, respectively, in 2014. However, especially at the end of the study period, prescriptions without indication codes decreased **(Figure 1)**.

To control for improvement of coding over time, we studied the total number of nitrofurantoin prescriptions, which in the Netherlands is only prescribed for UTIs. In women more clearly than in men, we see a flattening or even decrease of the number of non-coded nitrofurantoin prescriptions, whereas the number of coded and total nitrofurantoin prescriptions increased (**Figure S1**). Moreover, we calculated the proportion of antimicrobial drug prescriptions for UTIs with all antimicrobial drug prescriptions with an indication code (for all indications) as denominator. In this analysis, the proportion of antimicrobial drugs for UTIs increased from 5.2% in 1996 to 14% in 2014 for men and from 28% in 1996 to 50% in 2014 for women (**Figure S2**).

Choice of antimicrobial drug prescription in relation to the recommendations of the national primary care guidelines

Fluoroquinolones (J01MA) are the most commonly prescribed antimicrobial drugs in men with UTIs with no clear increase or decrease over time. Other frequently prescribed antimicrobial drugs were combinations of sulfonamides and trimethoprim (J01EE) and combinations of penicillins, including beta-lactamase inhibitors (J01CR); the last group increased significantly over time until 2013. Also, in men, the number of prescriptions of nitrofuran derivatives increased significantly from 0.4 prescriptions per 1000 PY in 1996 to 6.2 prescriptions per 1000 PY in 2014. In women, nitrofuran derivatives (J01XE) were clearly the most frequently prescribed drugs since 1999, with a strong and significant increase in the last years from 52 prescriptions per 1000 PY in 2008 to 98 prescriptions per 1000 PY in 2014. They increasingly replaced the prescriptions of trimethoprim and derivatives (J01EA) and fluoroquinolones (J01MA), which

were also commonly prescribed in women. Also, the prescriptions of combinations of penicillins, including beta-lactamase inhibitors (J01CR) and fosfomycin, significantly increased, for fosfomycin mainly in most recent years (**Figure 2**).

The implementation of the Dutch guideline on the treatment of UTIs of 2005 was associated in women with a significant decrease in the slope of prescriptions of trimethoprim and derivatives (J01EA) and an increase in nitrofuran derivative (J01XE) prescriptions. Unfortunately, we did not

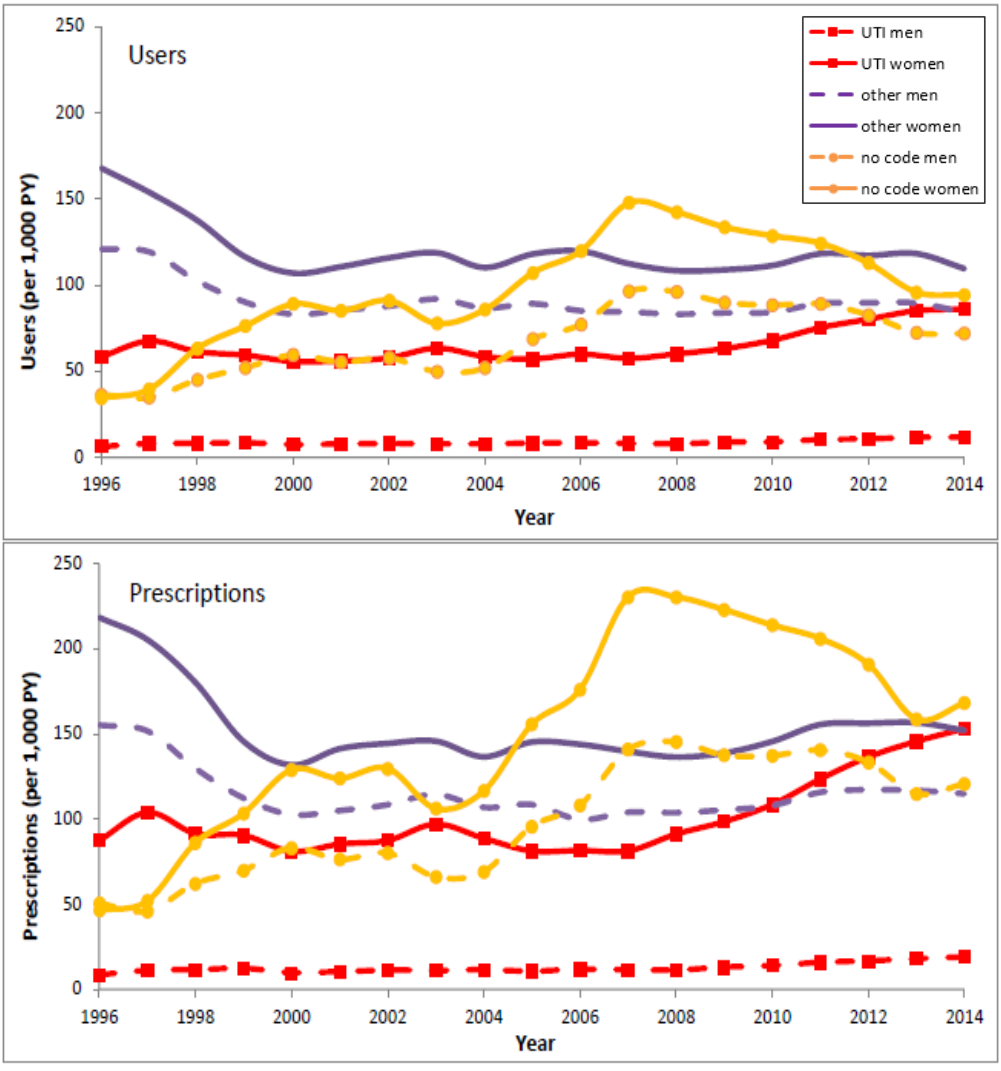


Figure 1: The number of users and prescriptions of antimicrobial drugs for urinary tract infections, other infections and infections without indication code in the period 1996-2014.

have sufficient data before 1999 and after 2013 to study the implementation of the other guidelines. For men, no significant effects were found of the implementation of the guidelines on the slope of any antimicrobial drug prescriptions.

Prescriptions of antimicrobial drugs for UTIs by age groups

Both in men and women, an increase in the total number of UTI prescriptions was observed for increasing age category. In women, the number of prescriptions in all age groups is higher than that in men, with nitrofuran derivatives (J01XE) as the most prescribed antimicrobial drug in all age groups, followed by fluoroquinolones (J01MA) and trimethoprim and derivatives (J01EA). In men, fluoroquinolones (J01MA) were the most frequently prescribed antimicrobial drugs in most age groups, followed by nitrofuran derivatives (J01XE) and combinations of penicillins, including beta-lactamase inhibitors (J01CR) (**Figure 3**). Analyzing the total number of prescriptions for UTIs (J01—antibacterials for systemic use) per age category, we observed an increase in the past years for all age categories, but most prominent in the higher age categories (**Figure S3**).

Changes in duration of nitrofurantoin prescriptions

In total, 215,531 prescriptions of nitrofurantoin with an ICPC code for UTIs were prescribed to women in the study period. In line with a change in recommended duration from 3 to 5 days in 2005, the proportion of prescriptions with a duration of 5 days increased strongly from 2005 onwards, while the proportion of prescriptions with a duration of 3 days became scarce. The proportion of prescriptions with a duration of 7 days decreased from 33% in 1996 to 18% in 2014. The proportion of prescriptions with a duration other than 3, 5 or 7 days (defined as 'other duration') remained stable over time, with a proportion ranging between 6% and 10% (**Figure 4**).

Discussion

In this study, we investigated the prescribing of antimicrobial drugs for UTIs in primary care during the study period from 1 January 1996 to 1 January 2015 and compared this with the recommendations in the Dutch primary care guidelines. These guidelines are actively distributed by the Dutch College of General Practitioners.

During the study period, nitrofurantoin was shown to be the most prescribed drug for the treatment of UTIs, especially in women, and increasingly in men. The Dutch guidelines of 1989 indicated nitrofurantoin as one of the options for women, whereas it was suggested as option

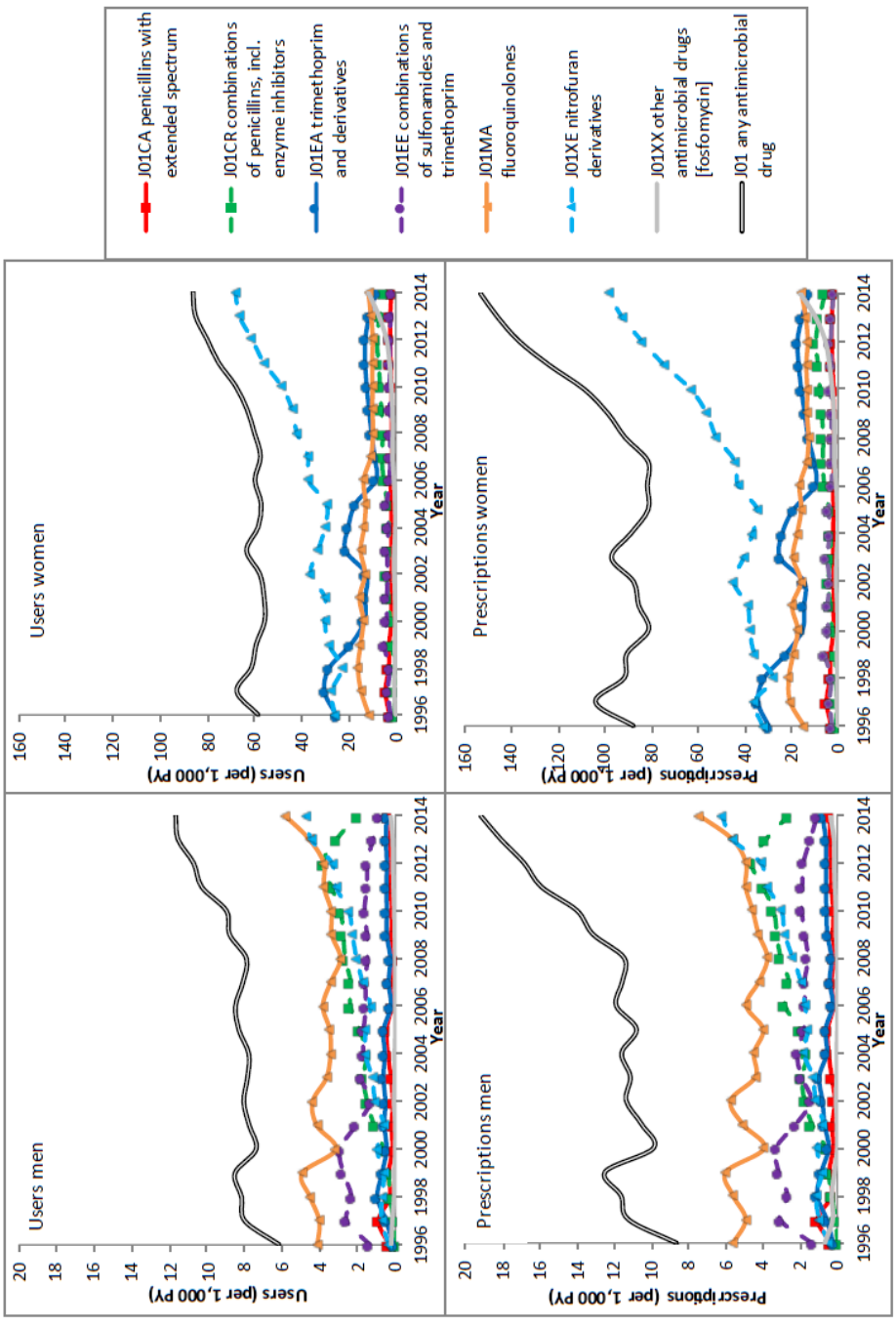


Figure 2: The number of users and prescriptions of antimicrobial drugs for urinary tract infections in the period 1996-2014.
Note that the scale of the y-axis differs between men and women.

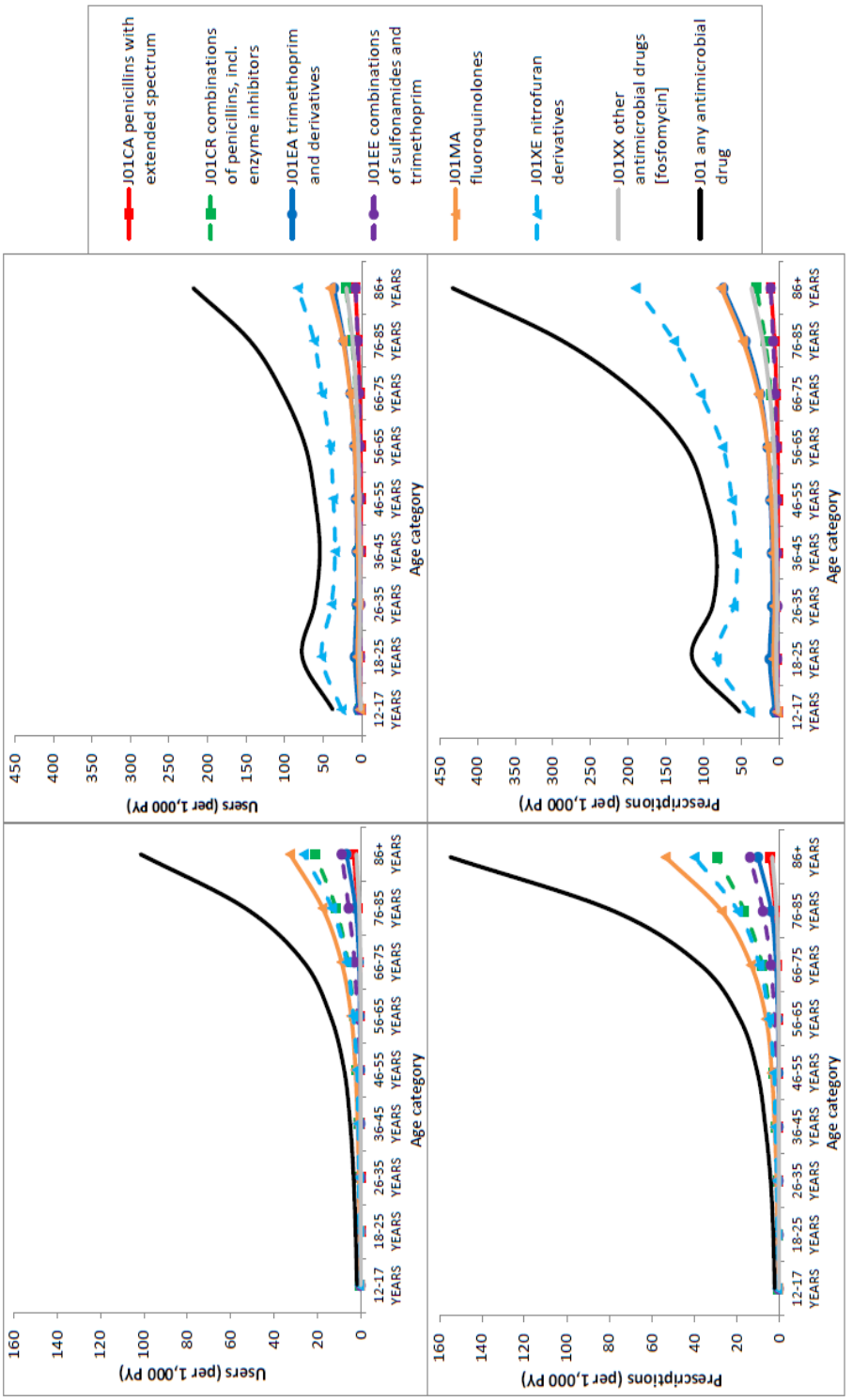


Figure 3: The number of users and prescriptions of antimicrobial drugs for urinary tract infections per age group. Note that the scale of the y-axis differs between men and women.

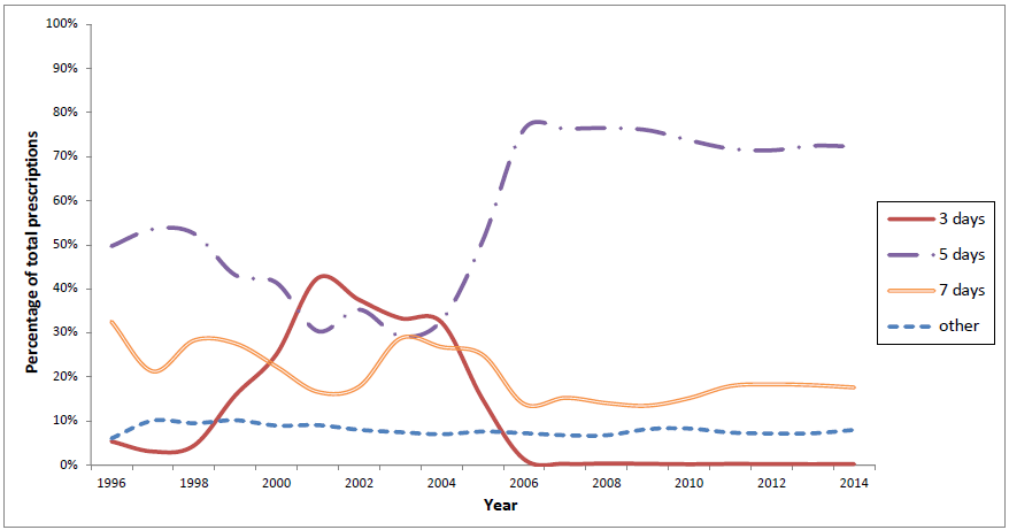


Figure 4: The proportion of the different durations of nitrofurantoin prescriptions in women in the period 1996–2014. The proportion of prescriptions of nitrofurantoin (J01XXE01) with a duration of 3, 5 and 7 days or 'other' duration (unequal to 3, 5 and 7 days or unknown) with all these nitrofurantoin prescriptions as denominator. Until 2005, the duration recommended by the guideline was 3 days, whereas since the guideline of 2005, the advised duration was 5 days.

for both men and women in the guideline of 1999 and as the first-choice drug for cystitis in the 2005 and 2013 guideline. This is reflected in our findings; we observed an increase in nitrofurantoin prescriptions in men and women since 1999, with a steep and significant increase since 2005. In this period, nitrofurantoin replaced trimethoprim in women, which significantly decreased after 2005. Furthermore, in the guidelines of 1999 and 2005, amoxicillin–clavulanic acid was recommended for UTIs with tissue invasion, whereas amoxicillin (or trimethoprim in men) was recommended for UTIs with tissue invasion in the guideline prior to 1999. In men and women, a significant increase of combinations of penicillins, including beta-lactamase inhibitors such as amoxicillin–clavulanic acid, was shown during the study period. In men, a flattening was seen from 2012, just before the guideline of 2013, which recommended ciprofloxacin as first choice.

We also studied the compliance of GPs to guidelines with regard to the duration of nitrofurantoin prescribing in women. The recommended duration increased from 3 to 5 days in 2005, which is reflected in our findings, indicating an increase in adherence to the guidelines by GPs, mainly since the guidelines of 2005 and 2013.[4–7] A deviation from the UTI guidelines was observed with regard to the prescribing of fluoroquinolones. Fluoroquinolones, in particular, ciprofloxacin, were only advised as first-choice drug for UTIs with tissue invasion from 2013 on (in 2005, it was second choice, and in 1989, its use was discouraged), whereas in men, these drugs were the

most frequently described antimicrobial drug during the complete study period. In women, we saw a significant decrease over time, suggesting an increase of compliance of GPs over time.

A study in another Dutch GP database investigated antimicrobial prescribing for several infections during 2001 and showed that approximately 75% of the prescriptions for cystitis were first-choice drugs (nitrofurantoin or trimethoprim) according to guidelines.[13] The percentage of first-choice drug prescriptions in our study in 2001 is comparable (approximately 67% for women). A smaller study from 2009 in 970 non-pregnant women of ≥ 11 years with cystitis determined that 66% were prescribed nitrofurantoin, whereas we found a proportion of 57% in all women in 2009.[14] Our proportion is somewhat smaller, possibly because we did not distinguish between cystitis and pyelonephritis, since a proportion of the prescriptions had an indication code for both. However, the sample size of the other study is much smaller, resulting in more uncertainty around the true prevalence of antimicrobial drug use.

As part of new health quality standards, GPs are encouraged to improve disease coding, a phenomenon also observed in other countries. This might in part explain the increase in total prescriptions for UTIs, which is underlined by our finding that the number of prescriptions for nitrofurantoin without an indication code seemed to decrease, especially in women since approximately 2007. To control for this bias, we analyzed UTI prescriptions as a proportion of the total number of antimicrobial drug prescriptions with an indication code (thus excluding prescriptions without a disease code). Yet, we observed that the increase of prescriptions was larger for UTIs than for other indications. This increase in antimicrobial drug prescribing for the treatment of UTIs is supported by other literature. According to the NIVEL (Netherlands Institute for Health Services Research) registration, the number of contacts with a GP for UTIs increased (especially in women) from 185 per 1000 in 2012 to 295 per 1000 in 2015.[3] This was confirmed by another large Dutch GP database, showing an increase of GP visits for UTIs and antimicrobial drug prescriptions for UTIs in the period 2007–2010.[15 16] This increase of use of antimicrobial drugs for UTIs might partly be explained by ageing of the population. Indeed, we showed that antimicrobial drugs for UTIs were especially prescribed to patients in older age categories. Additionally, a Dutch study from 2012 also showed an increase of antimicrobial drug prescribing by age, and an increase of total prescriptions of antimicrobial drugs to elderly: in 2000, 9% of patients above 80 years had at least one prescription, which increased to 22% in 2009.[8] However, ageing of the population cannot solely explain the increase, since we also showed an increase in prescriptions of antimicrobial drugs in younger age groups. Possible explanations might be that more patients suffer from recurrent UTIs, that patients more frequently visit their GP in case of a UTI or that GPs more easily prescribe antimicrobial drugs for UTIs, but this has to be elucidated in future studies.

One of the strengths of this study is that we used a large population-based cohort from a database with detailed information on prescriptions and where information is collected as part

of routine clinical care, reducing the risk of selection and information bias. We used these data to show that they can be of importance in studying antibiotic stewardship. Unfortunately, because of confidentiality, we could not study differences in prescribing patterns by region nor by GP characteristics such as age and/or gender. Additionally, although we found that antimicrobial drugs were increasingly prescribed according to the guidelines, the study design did not allow us to investigate the reasons behind deviations from the guidelines. This is of interest and should be studied further, eventually by qualitative prospective research to distinguish between forced deviations caused by resistant pathogens and other deviations, which should be diminished to promote good antimicrobial stewardship.

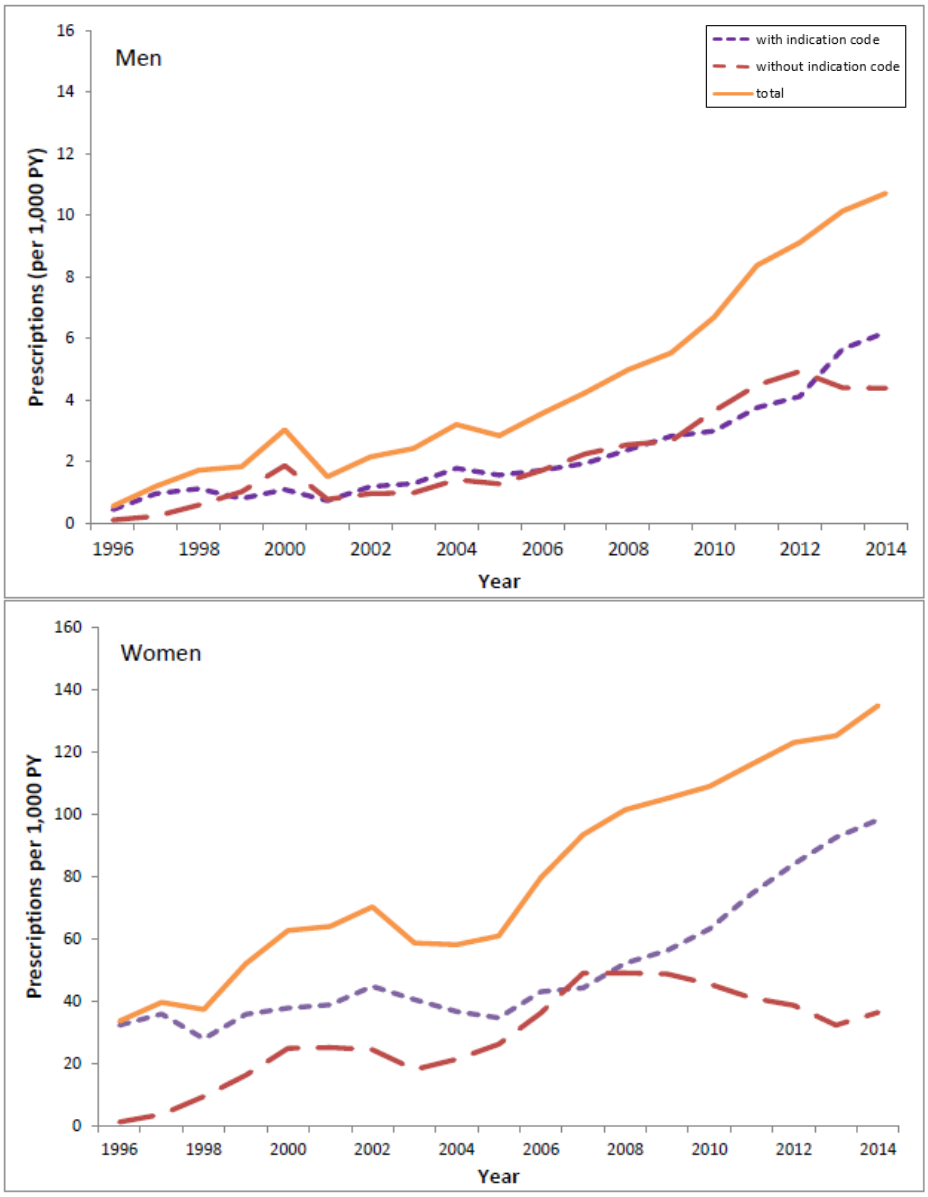
Reducing the use of antimicrobial drugs is important, since the drawback of treating infections with antimicrobial drugs is the development of AMR.[17] Patients suffering from infections caused by resistant bacteria have an increased risk of a worse clinical outcome and death.[17] Moreover, AMR leads to an increase in healthcare costs, which has been estimated to be 1.5 billion euros in EU every year.[18 19] Misuse and overuse of antimicrobial drugs are the most important risk factors of AMR worldwide.[20] Therefore, antimicrobial stewardship programmes have been developed, aiming to optimise the prescribing of antimicrobial drugs and to minimise misuse and overuse in order to minimise AMR.[21] Our study demonstrates that databases with antimicrobial drug prescriptions and indications are useful for surveillance of antimicrobial drug prescriptions over time. In future, information gathered in such databases should be provided to GP practices as part of antibiotic stewardship in primary care.

Supplementary materials

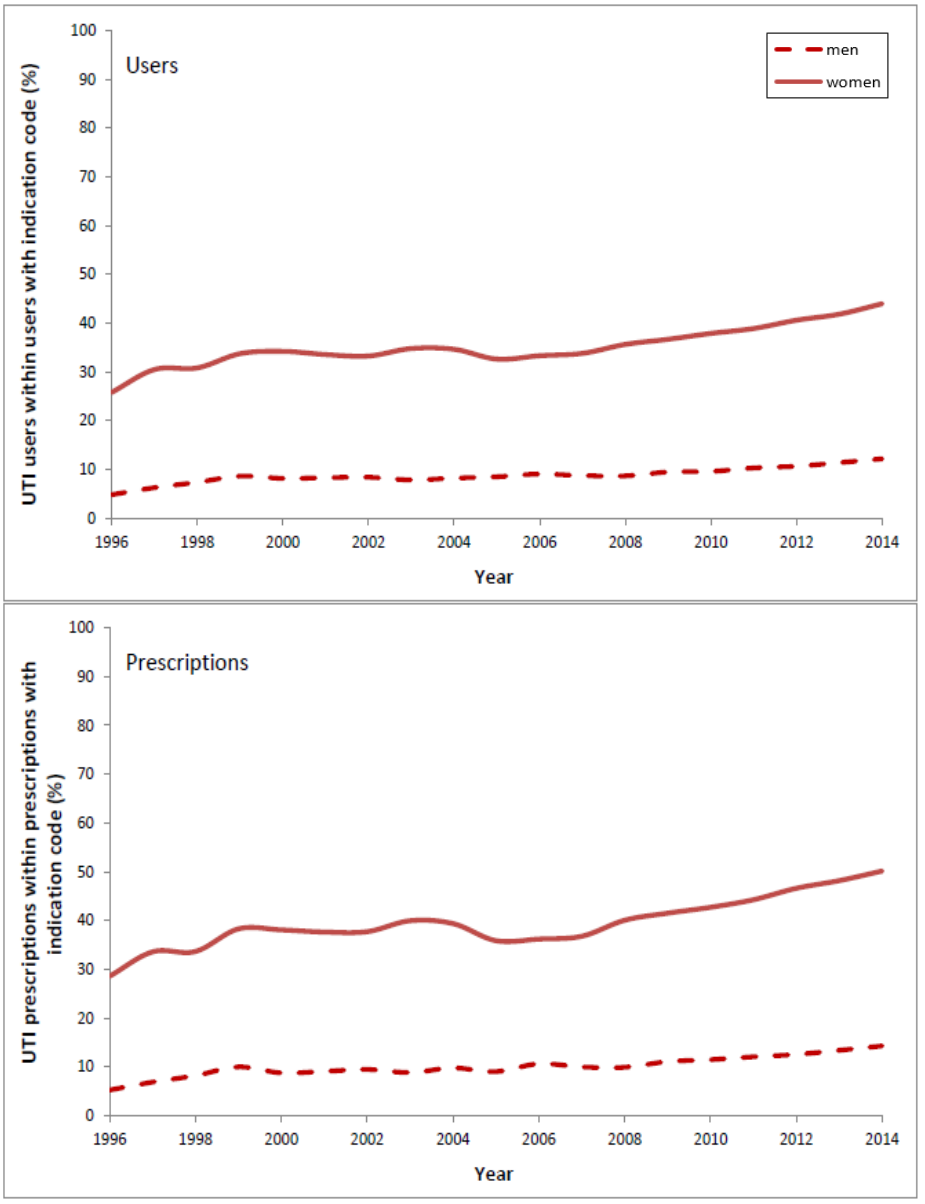
Supplementary table 1: ATC codes of antimicrobial drugs prescribed by GPs to treat urinary tract infections

ATC code	Drug class
J01AA*	Tetracyclines
J01CA	Penicillins with extended spectrum
J01CE*	Beta-lactamase sensitive penicillins
J01CF*	Beta-lactamase resistant penicillins
J01CR	Combinations of penicillins, incl. beta-lactamase inhibitors
J01DB*	First-generations cephalosporins
J01DC*	Second-generation cephalosporins
J01DD*	Third-generation cephalosporins
J01EA	Trimethoprim and derivatives
J01EB*	Short-acting sulfonamides
J01EE	Combinations of sulfonamides, and trimethoprim, incl. derivatives
J01FA*	Macrolides
J01FF*	Lincosamides
J01GB*	Other aminoglycosides
J01MA	Fluoroquinolones
J01MB*	Other quinolones
J01XC*	Steroid antibacterials
J01XE	Nitrofuran derivatives
J01XX	Other antibacterials

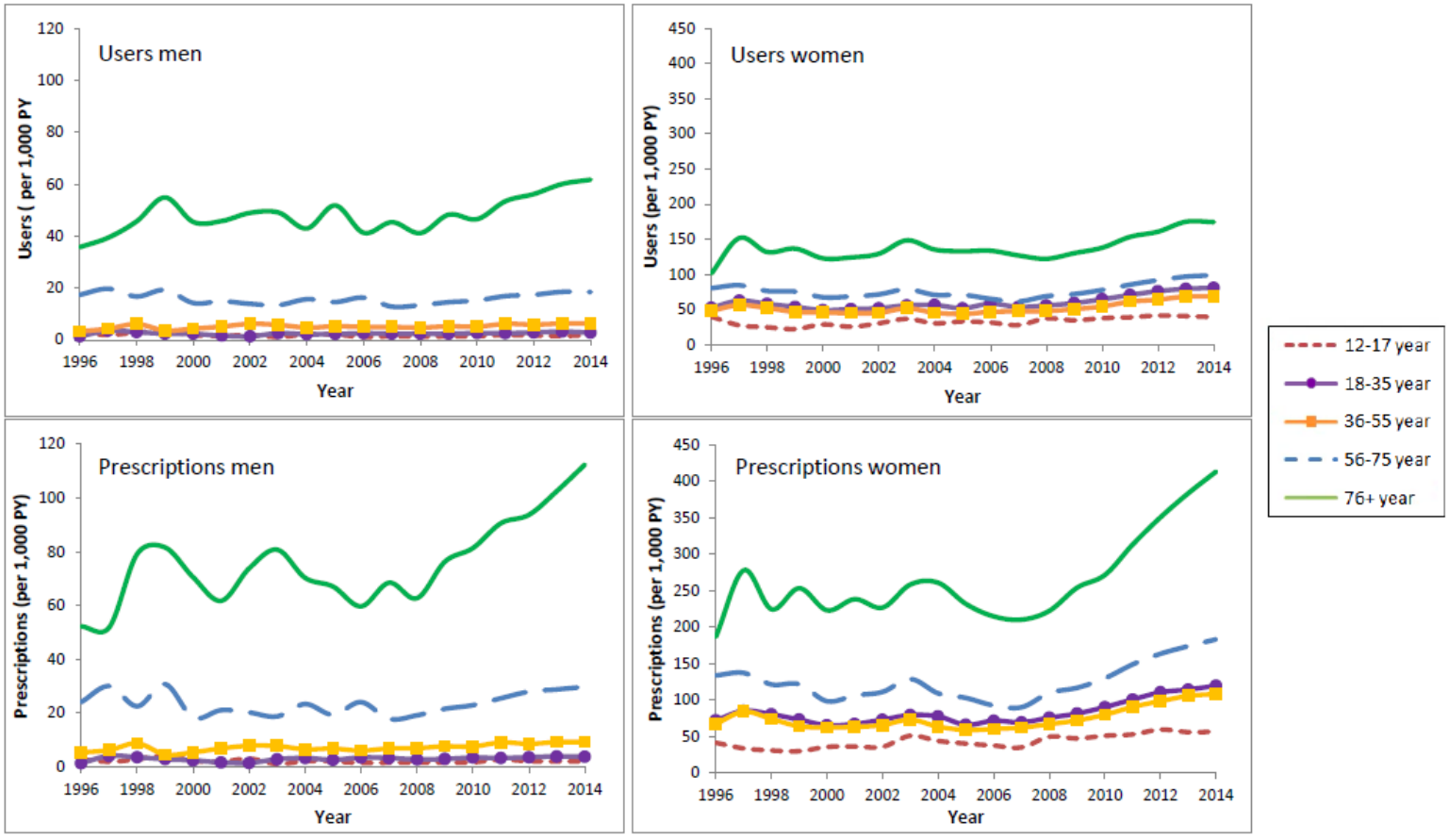
All antimicrobial drug classes that were prescribed for UTIs in the study population. The number of prescriptions of drugs with *was very low and are not shown in the figures.



Supplementary figure 1: The number of prescriptions of nitrofurantoin with and without an indication code for urinary tract infections for men and women.
Note that the scale of the y-axis differs between men and women.



Supplementary figure 2: The percentage of users/prescriptions with an indication code for urinary tract infections within all users/prescriptions with an indication code (thus including codes for urinary tract infections, airway infections, ear infections, skin infections etc.)



Supplementary figure 3: The number of users and prescriptions of antimicrobial drugs for urinary tract infections for different age groups per year. Note that the scale of the y-axis differs for men and women.

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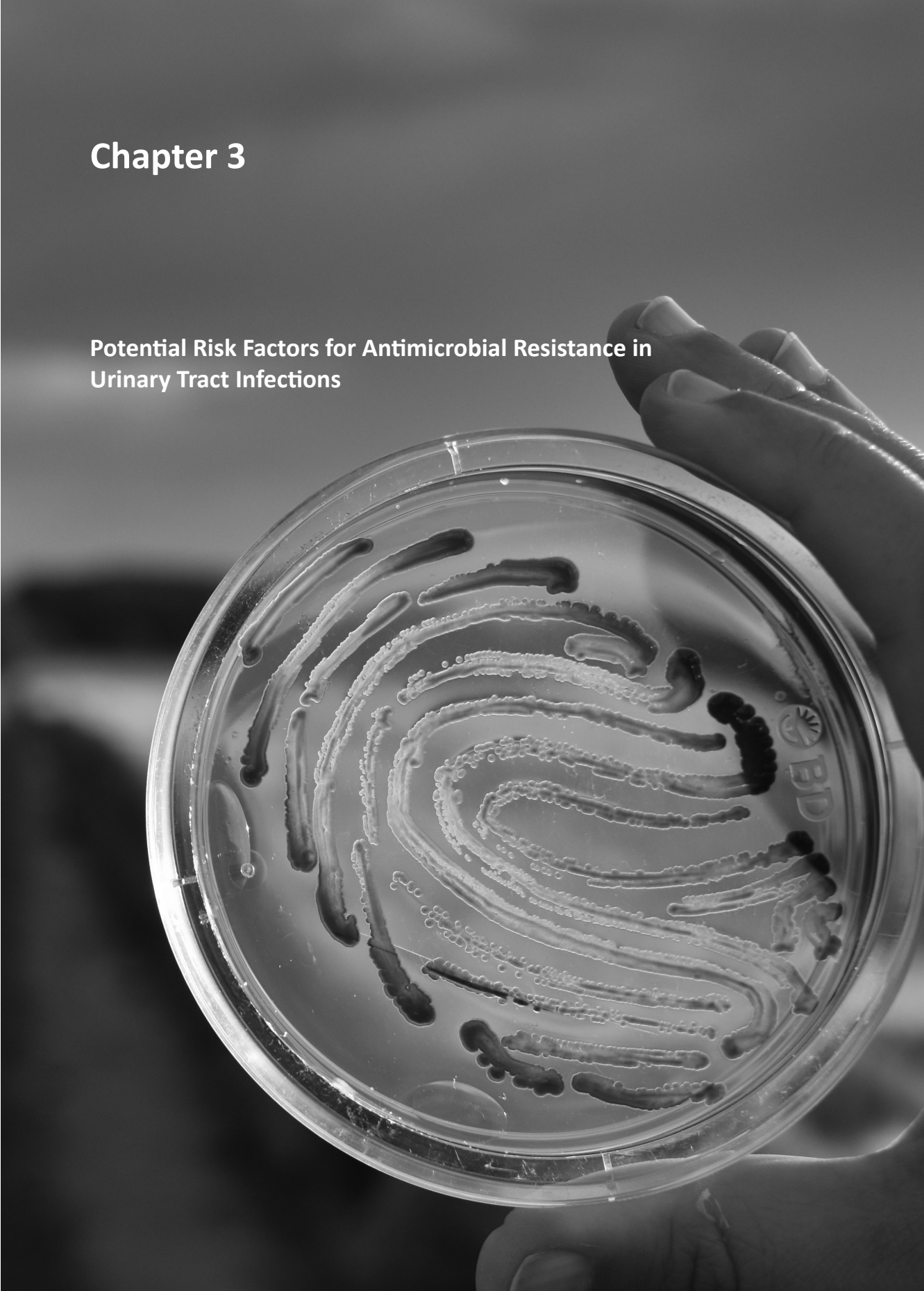
Chapter 3

Potential Risk Factors for Antimicrobial Resistance in Urinary Tract Infections



Chapter 3.1

Risk Factors for Resistance to Ciprofloxacin in Community-Acquired Urinary Tract Infections due to *Escherichia Coli* in an Elderly Population

Marlies Mulder, Jessica Kiefte-de Jong, Wil Goessens,, Herman de Visser, Albert Hofman, Bruno Stricker, Annelies Verbon

J Antimicrob Chemother. 2017 Jan;72(1):281-289.

Abstract

Background Antimicrobial resistance to ciprofloxacin is rising worldwide, especially in bacteria causing urinary tract infections (UTIs). Prudent use of current antibiotic drugs is therefore necessary.

Objective We analyzed (modifiable) risk factors for ciprofloxacin-resistant *Escherichia coli*.

Methods Urinary cultures of UTIs caused by E. coli were collected from participants in the Rotterdam Study, a prospective cohort study in an elderly population, and analyzed for susceptibility to ciprofloxacin. Multivariate logistic regression was performed to investigate several possible risk factors for resistance.

Results Ciprofloxacin resistance in 1080 E. coli isolates was 10.2%. Multivariate analysis showed that higher age (OR 1.03; 95% CI 1.00-1.05) and use of two (OR 5.89; 95% CI 3.45-10.03) and three or more (OR 3.38; 95% CI 1.92-5.97) prescriptions of fluoroquinolones were associated with ciprofloxacin resistance, while no association between fluoroquinolone use more than 1 year before culture and ciprofloxacin resistance could be demonstrated. Furthermore, a high intake of pork (OR 3.68; 95% CI 1.36-9.99) and chicken (OR 2.72; 95% CI 1.08-6.85) and concomitant prescription of calcium supplements (OR 2.51; 95% CI 1.20-5.22) and proton pump inhibitors (OR 2.04; 95% CI 1.18-3.51) were associated with ciprofloxacin resistance.

Conclusions Ciprofloxacin resistance in community-acquired UTI was associated with a high intake of pork and chicken and with concomitant prescription of calcium supplements and proton pump inhibitors. Modification of antibiotic use in animals as well as temporarily stopping the prescription of concomitant calcium and proton pump inhibitors need further evaluation as strategies to prevent ciprofloxacin resistance.

Introduction

Urinary tract infections (UTIs) are common in women, especially after menopause. Nearly 50% of all women experience at least one UTI during their lifetime.[1] Fluoroquinolones are often prescribed for complicated UTIs, such as pyelonephritis.[2] However, ciprofloxacin, for example, is also widely used in uncomplicated UTIs.[3]

With the increasing use of fluoroquinolones, selection of ciprofloxacin-resistant uropathogens has become widespread worldwide, ranging from 2% to 69% for uncomplicated and up to 98% for complicated UTIs.[4] In Europe, resistance to ciprofloxacin in uncomplicated UTIs varied from 4.8% in France, 20.3% in Germany, 30.8% in Spain, 7.3% in Sweden to 15.3% in the UK in 2014,[5] while in the USA 17.1% resistance to ciprofloxacin was seen in an outpatient population with UTIs.[6] Although antimicrobial resistance (AMR) is low in the Netherlands, resistance of *Escherichia coli* to ciprofloxacin in outpatients was ~10% in 2014,[7] while it was 12% in a study with a mixed population of both inpatients and outpatients with complicated UTIs in 2004–09.[8]

The WHO has proclaimed AMR to be one of the greatest current threats to global health. Furthermore, AMR is directly associated with the use of antibiotics.[9] Unfortunately, there are very few new antibiotic drugs in the pipeline,[10] and prudent use of current antibiotic drugs has been advocated in order to decrease AMR rates.[11] This also involves dose adjustment to obtain proper drug levels, since low levels of ciprofloxacin will influence the mutant selection window, resulting in faster selection of resistant subpopulations.[12]

Age and renal insufficiency influence the concentrations of ciprofloxacin in blood and urine,[13,14] and furthermore concomitantly prescribed drugs may change the pharmacokinetics of ciprofloxacin, such as bioavailability or excretion. Taking age and renal function into account or adjusting for co-medication may result in improved drug levels and less selection of ciprofloxacin-resistant bacteria. However, other modifiable factors may also play a role. Therefore, we studied risk factors for ciprofloxacin-resistant *E. coli* in a primary care population with UTI and identified modifiable factors that were associated with ciprofloxacin resistance.

Patients and methods

Source population

This nested case–control analysis was embedded in the Rotterdam Study, which was designed as a prospective population-based cohort study of chronic diseases as a response to demographic changes that lead to an increase of elderly people in most populations. The aim of the study is to investigate determinants of disease occurrence and progression in the elderly and to investigate potentially modifiable determinants in order to be able to develop preventive

strategies. In 1990 all inhabitants aged 55 years and older living in the well-defined Ommoord district in Rotterdam, the Netherlands were invited to participate (response rate 78%). In an extension in 2000 and 2006, two further cohorts were included with inhabitants aged 55 years and older, and 45 years and older, respectively, resulting in a total of 14.926 participants. All participants are invited every 3–4 years for follow-up interviews and examinations. The majority of people included in the study cohort are of Dutch ancestry and are representative of the Dutch population at that age.[15,16]

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, on the basis of the Wet Bevolkingsonderzoek ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent for their participation in the study and for researchers to obtain information from their treating physicians.

Cases and controls

In the Rotterdam area all laboratory services for community-dwelling patients of general practitioners are provided by Star Medisch Diagnostisch Centrum (Star-MDC). The study population consisted of participants from the Rotterdam Study with at least one urinary culture positive for *E. coli* between 1 January 2000 and 31 January 2014. Cultures and uricult slides were collected from midstream urine. A standardized volume of urine was cultured on agar plates and incubated overnight at 35°C; the same was done for colonies of microorganisms from uricult slides. A culture was considered positive when at least 10^3 cfu/mL were present. Susceptibility to ciprofloxacin was until 2010 determined by disc diffusion, and afterwards with the VITEK 2 system (VITEK AMS; bioMérieux Vitek Systems, Inc., Hazelwood, MO, USA). Until 2013 the CSLI criteria[15] were used for zone size and MIC interpretations of ciprofloxacin. After 2013 the EUCAST criteria[16] were applied. In our study intermediate results for ciprofloxacin were categorized as resistant. All isolates after 2013 that were resistant to ciprofloxacin had an MIC >4 mg/L. Only the first culture positive for *E. coli* of each patient, and thus one culture per patient in total, was used for the analysis. Cases were defined as participants with a positive urine culture with ciprofloxacin-resistant *E. coli*; controls were participants with a positive urine culture with ciprofloxacin-susceptible *E. coli*.

Determinants

Characteristics of participants have been collected in the Rotterdam Study at baseline. BMI was calculated and categorized into three categories: normal (18.5–25.0 kg/m^2), underweight (<18.5 kg/m^2) and overweight (>25.0 kg/m^2). Serum creatinine, taken as the closest assessment preceding the date of urinary culture, was obtained from Star-MDC and used to calculate the glomerular filtration rate, according to the CKD-EPI equation. If absent, serum creatinine from visits to the study center was obtained. Kidney function was categorized into three categories:

good (>60 mL/min per 1.73 m^2), moderate (30–60 mL/min per 1.73 m^2) and poor (<30 mL/min per 1.73 m^2). Diabetes mellitus was defined as use of anti-diabetic medication. Socioeconomic status (SES) was scored according to the UNESCO criteria.

Medication use

Medication use of participants in the Rotterdam Study has been monitored since 1 January 1991, through computerized records from pharmacies in the Ommoord district. The data include the Anatomical Therapeutical Chemical code, dispensing date, total number of drug units per prescription, the prescribed daily number of units and the product names of drugs. The number of prescriptions of fluoroquinolones before first urinary culture since the beginning of pharmacy data collection was determined. It was separated into prescriptions before the start of the study period (historical use) and during this period. Both variables were categorized in none, one, two and three or more prescriptions. Prescriptions in the month before culture were not included, since it was assumed that the culture was sent in because of persisting symptoms despite fluoroquinolone use. The same information was collected for the antibiotic groups J01C (β-lactam antibacterials, penicillins), J01EA (trimethoprim and derivatives) and J01XE (nitrofuran derivatives), which was dichotomized into use or no use. Furthermore, the timing of the last fluoroquinolone prescription, defined as the time between last fluoroquinolone prescription and culture, was calculated and categorized into: no prescription; >12 months ago; 3–12 months ago; 1–3 months ago. Again, participants with a prescription in the month before culture were excluded from this variable. Concomitant drug use was defined as drug use during the 90 day period before filling the most recent prescription of any fluoroquinolone before culture. Investigated drugs were antacids (A02A); H_2-receptor antagonists (A02BA); proton pump inhibitors (A02BC); vitamins (A11); mineral supplements (A12); diuretics (C03); calcium channel blockers (C08); drugs acting on the renin–angiotensin system (C09) and corticosteroids for systemic use (H02). The main fluoroquinolones prescribed were ciprofloxacin (43%), norfloxacin (38%) and ofloxacin (15%).

Diet

By using an extensive semi-quantitative food frequency questionnaire (FFQ), the habitual food intake of participants of several cohorts of the Rotterdam Study was determined (2006–12). The questionnaire consisted of 389 food items and questions about dietary habits. This FFQ has been developed on the basis of an existing validated FFQ developed for Dutch adults.[17,18] Participants were asked to give for each food item the frequency of consumption (in times per month or per week), the number of servings per day (expressed in standardized household measures) as well as the preparation method. All food items were calculated to grams per day. Total energy intake was calculated using the Dutch Food Composition Table of 2006 and 2011. Participants who reported an extreme energy intake of <500 kcal/day or >5000 kcal/day were excluded *a priori* from analysis; 18 participants (3.4%) fell into this category. To account for potential

measurement error and confounding, dietary intake was adjusted for total energy intake, using the residual method. Briefly, a linear regression of every dietary variable was performed with total energy intake per day in calories as an outcome. Residuals were added to the coefficients of the formula times the median food intake per day of the population [residuals + (β0 + β1) × median calorie intake per day], resulting in dietary intake fixed to the median energy intake of the population, as explained by Willett *et al.*[19] The energy-adjusted dietary variables were categorized in tertiles.

Analysis and statistical methods

Multivariate binary logistic regression was performed with as an outcome variable the presence or absence of ciprofloxacin-resistant *E. coli* to determine the OR and 95% CI of several determinants. Variables for the multivariate models were considered on the basis of clinical grounds and based on the literature:[20–25] the number of prescriptions of fluoroquinolones before first culture during the study period, historical use of fluoroquinolones, timing of the last fluoroquinolone prescription, sex, age, kidney function, diabetes, BMI, SES, and a variable consisting of the period between the start date of the study and the date of culture (follow-up time). The number of prescriptions of fluoroquinolones (model 1) and the timing of the last fluoroquinolone prescription (model 2) were studied in separate models because of their intrinsic similarity. Variables were selected on the basis of clinical relevance, while backward selection ($P < 0.2$) was used to determine the final model. Because of clinical relevance, BMI, SES and Dutch Healthy Diet index (DHDI) were kept fixed in the models, while historical use of fluoroquinolones was not. These variables were separately studied in an extra analysis by removing them one by one from the model to see whether these would change the OR by >10%. Furthermore, use of other antibiotics and of concomitantly described drugs possibly affecting the pharmacokinetics of fluoroquinolones were also analyzed in model 1.

Dietary meat intake was analyzed in multivariate model 1, which included an additional score, the DHDI of each participant, which reflects the overall healthy diet pattern assessed by adherence to the Dutch Guidelines for a Healthy Diet, which was adapted from van Lee *et al.*[26] and included the following components: intake of fibers, vegetables, fruit, fish, saturated fatty-acids, mono-trans fatty-acids, sodium and alcohol. For each meat (beef, chicken, pork and offal) the effect of substituting one of the others was estimated by including simultaneously these meat types as continuous variables in the model. This was performed in order to exclude the possibility that the associations found were not true associations, but caused by a high correlation with the intake of another meat. The risk difference for the substitution effect was derived from the difference between the regression coefficients when the food items were added alone and simultaneously, where a small difference would indicate that this effect can be neglected.[27]

To account for potential bias associated with missing data, missing values on kidney function (2.0%), BMI (4.6%) and SES (1.4%) were imputed using multiple imputation ($n = 10$ imputations).[28] Details of the procedure are described in Table S1, while Table S2 (both available as Supplementary data at JAC Online) shows the characteristics of the study population before and after the procedure.

A *P* value <0.05 was considered as statistically significant. Statistical analyses were performed with IBM SPSS Statistics 21.

Results

The source population consists of 14,926 participants of the Rotterdam Study. From this population, 1999 urinary cultures positive for *E. coli* were collected from 1080 individuals. These individuals comprise the current study population. General characteristics are shown in **Table 1**. The study population consisted of more women (80.3%) compared with the total population of

the Rotterdam Study (59.1%). Ciprofloxacin-resistant *E. coli* was found in 10.2% of all urine cultures positive for *E. coli*. The percentage of resistant cultures did not change over the study period. In the 415 individuals with one or more prescriptions of fluoroquinolones before the first urinary culture, ciprofloxacin resistance was higher (18.3%). Thirty-four (30.9%) patients with a UTI caused by ciprofloxacin-resistant *E. coli* had no prescriptions of fluoroquinolones during the study period before culture compared with 631 (65.1%) of patients with a UTI caused by ciprofloxacin-susceptible *E. coli*. In the subgroup of participants with at least one prescription of a fluoroquinolone before culture, the median defined daily dose was 1.0 and the median duration was 7.0 days. Neither of these was significantly associated with ciprofloxacin resistance (data not shown).

Table 2 shows the multivariate models. Higher age (increase per year: OR 1.03; 95% CI 1.00–1.05), renal insufficiency (OR 2.22; 95% CI 1.04–4.78) and two (OR 5.89; 95% CI 3.45–10.03) and three or more (OR 3.38; 95% CI 1.92–5.97) prescriptions of fluoroquinolones during the study period were associated with ciprofloxacin-resistant *E. coli* in model 1. In model 2, higher age (OR 1.04; 95% CI 1.01–1.06), sex (OR 1.78; 95% CI 1.00–3.17) and prescriptions of a fluoroquinolone 3–12 months before culture (OR 4.38; 95% CI 2.15–8.93) and 1–3 months before culture (OR 4.69; 95% CI 1.97–11.13) were significantly associated with ciprofloxacin resistance.

A prescription of a fluoroquinolone more than 12 months before culture was not significantly associated. No significant associations were demonstrated for previous prescriptions of other antibiotic drugs, namely penicillins trimethoprim derivatives and nitrofuran derivatives (data not shown).

Table 1: General characteristics of the study population

	Total N = 1080	Cases N = 110	Controls N = 970
Sex, n (%)			
Male	213 (19.7)	28 (25.5)	185 (19.1)
Female	867 (80.3)	82 (74.5)	785 (80.9)
Age, median (range)	73 (65)	79 (52)	73 (65)
BMI, n (%)			
Normal (18.5 – 25.0 kg/m^2)	276 (25.6)	25 (22.7)	251 (25.9)
Underweight (<18.5 kg/m^2)	9 (0.8)	0 (0.0)	9 (0.9)
Overweight (> 25 kg/m^2)	745 (69.0)	80 (72.7)	665 (68.6)
Missing (%)	50 (4.6)	5 (4.5)	45 (4.6)
Kidney function, n			
Good (> 60 mL/min per 1.73 m^2)	683 (63.2)	51 (46.4)	632 (65.2)
Moderate (30 – 60 mL/min per 1.73 m^2)	315 (29.2)	41 (37.3)	274 (28.2)
Poor (<30 mL/min per 1.73 m^2) †	60 (5.6)	15 (13.6)	45 (4.6)
Missing (%)	22 (2.0)	3 (2.7)	19 (2.0)
Diabetes, n	154 (14.3)	25 (22.7)	129 (13.3)
SES, n			
Primary education	188 (17.4)	24 (21.8)	164 (16.9)
Lower/intermediate general education or lower vocational education	526 (48.7)	52 (47.3)	474 (48.9)
Intermediate vocational education or higher general education	246 (22.8)	25 (22.7)	221 (22.8)
Higher vocational education or university	105 (9.7)	8 (7.3)	97 (10.0)
Missing (%)	15 (1.4)	1 (0.9)	14 (1.4)
Number of prescriptions of fluoroquinolones, n (%)			
no prescriptions	665 (61.6)	34 (30.9)	631 (65.1)
1 prescription	163(15.1)	15 (13.6)	148 (15.3)
2 prescriptions	86 (8.0)	13 (11.8)	73 (7.5)
3 or more prescriptions	166 (15.4)	48 (43.6)	118 (12.2)
Timing last fluoroquinolone prescription, n			
no prescriptions	665 (61.6)	34 (30.9)	631 (65.1)
> 12 months before culture	236 (21.9)	21 (19.1)	215 (22.2)
3-12 months before culture	65 (6.0)	14 (12.7)	51 (5.3)
1-3 months before culture	41 (3.8)	9 (8.2)	32 (3.3)
0-1 months before culture	73 (6.8)	32 (29.1)	41 (4.2)
DDD of last pscription, median (range)	-	1.0 (2.0)	-
Duration of last prescription, median days (range)	-	7.0 (70.0)	-
At least 1 prescription of penicillins	794 (73.5)	86 (78.2)	708 (73.0)
At least 1 prescription of nitrofuran derivatives	587 (54.3)	60 (54.5)	527 (54.3)
At least 1 prescription of trimethoprim deratives	531 (49.2)	60 (54.5)	471 (48.6)

Data are median (range) for continuous or the number (%) for categorical variables, overall and separately for cultures resistant or susceptible to ciprofloxacin. BMI is body mass index in kg/m^2. Kidney function is glomerular filtration rate, according to the CKD EPI equation in mL/min per 1.73 m^2. SES is socioeconomic status, DDD is defined daily dose.

Table 2: Multivariate model for ciprofloxacin resistance in urinary tract infections due to *E.coli*

	OR (95% CI)	OR Model 1 (95% CI)	OR Model 2 (95% CI)
Age	1.04 (1.03 – 1.06) †	1.03 (1.00 – 1.05) †	1.04 (1.01 – 1.06) †
Sex (ref = female)	1.45 (0.92 – 2.29)	1.51 (0.91 – 2.51)	1.78 (1.00 – 3.17) †
BMI:			
Normal	ref	ref	ref
Underweight	-	-	-
Overweight	1.20 (0.74 – 1.93)	1.08 (0.64 – 1.80)	1.24 (0.68 – 2.27)
Kidney function:			
Good	ref	ref	ref
Moderate	1.87 (1.21 – 2.89) †	1.40 (0.87 – 2.26)	0.93 (0.52 – 1.65)
Poor	4.26 (2.24 – 8.11) †	2.22 (1.04 – 4.78) †	2.22 (0.95 – 5.19)
Diabetes	1.92 (1.18 – 3.11) †	1.57 (0.93 – 2.65)	0.96 (0.49 – 1.87)
FQ prescriptions:			
No prescriptions of FQ	ref	ref	-
1 prescription of FQ	1.92 (1.03 – 3.59) †	1.67 (0.88 – 3.16)	-
2 prescriptions of FQ	6.89 (4.10 – 11.60) †	5.89 (3.45 – 10.03) †	-
≥3 prescriptions of FQ	3.99 (2.30 – 6.93) †	3.38 (1.92 – 5.97) †	-
Timing of FQ prescription before culture:			
No prescription of FQ	ref	-	ref
>12 months	1.81 (1.03 – 3.19) †	-	1.50 (0.84 – 2.68)
3-12 months	5.10 (2.57 – 10.10) †	-	4.38 (2.15 – 8.93) †
1-3 months	5.22 (2.31 – 11.81) †	-	4.69 (1.97 – 11.13) †

Odds ratios (OR) and 95% confidence intervals (95% CI) for several determinants of ciprofloxacin resistance in urinary cultures both unadjusted as adjusted in two multivariate models. FQ = fluoroquinolone; BMI = Body Mass Index in kg/m^2 with normal is 18.5-25.0 kg/m^2, underweight is <18.5 kg/m^2 and overweight is >25 kg/m^2. Kidney fuction = glomerular filtration rate estimated with the CKD-EPI equation in mL/min per 1.73 m, with good is >60mL/min per 1.73m^2, moderate is 30-60 mL/min per 1.73 m^2 and poor is <30 mL/min per 1.73m^2. In model 1 are included: age, sex, BMI, kidney function, diabetes, socioeconomic status, follow-up time and number of fluoroquinolone prescriptions. In model 2 are included: age, sex, BMI, kidney function, diabetes, socioeconomic status, follow-up time and the timing of last fluoroquinolone prescription. 73 (6.8%) particpants were excluded in model 2 because they had a presciption of a fluoroquinolone in the first month before culture. In model 1 the prescriptions in the first month before culture were not included in the total number. † significant with p <0,05.

Dietary intake of several types of meat (beef, chicken, offal and pork) and having a urinary culture with a ciprofloxacin-resistant *E. coli* was investigated in a subgroup of 507 individuals for which data on nutritional intake had been collected. In model 1, high intake of pork (highest tertile versus lowest tertile: OR 3.68; 95% CI 1.36–9.99; trend: OR 1.97; 95% CI 1.19–3.25) and a high intake of chicken (highest versus lowest tertile: OR 2.72; 95% CI 1.08–6.85; trend: OR 1.67; 95% CI 1.05–2.65) were associated with ciprofloxacin resistance after adjustment for confounders

(**Table 3**). Substituting beef with pork showed some effect on the association with ciprofloxacin resistance; however, this effect was not significant (data not shown). No effects were seen for the other kinds of meat.

The concomitant prescription of other drugs during ciprofloxacin prescription is shown in **Table 4**. After adjustment for age, sex, kidney function, diabetes, BMI, SES and follow-up time a significant association could be demonstrated for mineral supplements (OR 2.51; 95% CI 1.20–5.22), which mainly consisted of calcium supplements, and for proton pump inhibitors (OR 2.04; 95% CI 1.18–3.51).

Discussion

In this study, we investigated the association of modifiable determinants and the selection of ciprofloxacin-resistant *E. coli* causing UTI in an elderly community-based cohort. Risk factors such as prior fluoroquinolone use and higher age were present, whereas other reported risk factors such as diabetes were not significant in our study. Furthermore, a high intake of pork and chicken as well as the concomitant prescription of calcium supplements or proton pump inhibitors with fluoroquinolones were associated with the selection of ciprofloxacin-resistant *E. coli.*

In this community-dwelling cohort of mainly elderly women, concomitant prescription of fluoroquinolones and calcium supplements is likely since both recurrent UTIs and the use of calcium supplements are common in postmenopausal women, as described previously in an inpatient population.[29] Calcium, as well as compounds containing magnesium and aluminum, belong to the group of di- or trivalent cation-containing compounds (DTCCs), which have been shown to have effects on the bioavailability of fluoroquinolones.[30] They are known to change the pharmacokinetics of several fluoroquinolones by diminishing their solubility and thereby decreasing absorption, resulting in an increase in excretion through the intestines, lower blood levels and lower urine levels.[31–33] Proton pump inhibitors, such as omeprazole, have also been found to diminish the bioavailability of fluoroquinolones, probably by increasing gastric pH,[34,35] although this could not be confirmed in another study.[36] Pharmacokinetic studies have suggested a higher risk of resistance when blood levels of ciprofloxacin are low.[37] Indeed, concomitant use of levofloxacin and DTCCs has been shown to increase selection of fluoroquinolone-resistant bacteria.[38] Here, we showed that concomitant prescription of calcium supplements and proton pump inhibitors with fluoroquinolones was associated with ciprofloxacin-resistant *E. coli.*

High intake of pork and high intake of chicken may also be potential risk factors. For chicken, it is known that a substantial amount of *E. coli* isolates are present in samples of raw meat in the Netherlands. This is in contrast to pork, where these percentages are low.[7] In addition,

Table 3: Ciprofloxacin resistance and meat intake

	Tertile of intake			Trend
	1	**2**	**3**	
Beef				
Median intake (gr/day)	11.9	30.0	57.1	
Cases	11	13	12	36
OR (95% CI)	ref	1.20 (0.52 – 2.75)	1.10 (0.47 – 2.56)	1.05 (0.69 – 1.58)
Adjusted OR (95% CI)	ref	1.38 (0.55 – 3.48)	1.10 (0.43 – 2.80)	1.04 (0.66 – 1.64)
Pork				
Median intake (gr/day)	13.9	35.4	65.4	
Cases	7	10	19	36
OR (95% CI)	ref	1.46 (0.54 – 3.92)	2.93 (1.20 – 7.17) †	1.76 (1.13 – 2.74) †
Adjusted OR (95% CI)	ref	1.57 (0.56 – 4.47)	3.68 (1.36 – 9.99) †	1.97 (1.19 – 3.25) †
Offal				
Median intake (gr/day)	0.0	0.0	5.9	
Cases	10	8	18	36
OR (95% CI)	ref	0.79 (0.30 – 2.05)	1.90 (0.85 – 4.24)	1.44 (0.94 – 2.21)
Adjusted OR (95% CI)	ref	0.73 (0.26 – 2.03)	1.78 (0.72 – 4.41)	1.40 (0.87 – 2.25)
Chicken				
Median intake (gr/day)	0.0	9.3	37.1	
Cases	11	9	16	36
OR (95% CI)	ref	0.81 (0.33 – 2.00)	1.50 (0.68 – 3.34)	1.25 (0.82 – 1.91)
Adjusted OR (95% CI)	ref	1.41 (0.51 – 3.86)	2.72 (1.08 – 6.85) †	1.67 (1.05 – 2.65) †

Odds Ratios (OR) and 95 % confidence intervals (95% CI) for resistance to ciprofloxacin in urinary cultures for several meat items. Median intake is the median unadjusted intake of the meat in grams per day. Intake of all meat items was adjusted for total energy intake by taking the residuals after lineair regression and categorised into tertiles. ORs were adjusted for age, sex, BMI, kidney function, diabetes, SES, follow-up time, DHD index and number of prescriptions of fluoroquinolones. † significant with p <0,05. A sensitivity analysis was performed by removing BMI, SES, follow-up time and DHDI separately from the model, which showed no changes to the OR larger than 10%.

substitution of other meats could not explain the association between high pork intake and ciprofloxacin resistance. An association between antibiotic use in animals and the resistance rate of Gram-negative organisms isolated in humans was first described in the 1960s,[39] followed by a number of other reports.[40–42] Furthermore, a recent population-based study on the gut microbiota showed a correlation between meat consumption and overall microbiome community variation.[43] Evidence supporting transmission of microorganisms from retail meat to humans has been reported for extended-spectrum β-lactamase-producing *E. coli.*[44] Although AMR was higher in meat source *Klebsiella pneumoniae* than in phylogenetically closely related clinical isolates,[41] this does not exclude the possibility of transmission, since transmission of plasmids carrying colistin resistance genes from *E. coli* to *K. pneumoniae* has been made likely.[45]

Table 4: Ciprofloxacin resistance and concomitantly prescribed drugs.

Concomitantly prescribed drugs	Medication use in resistant cultures, n (%)	Medication use in susceptible cultures, n (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
H_2-receptor antagonists	3 (3.9)	13 (3.8)	1.03 (0.29 – 3.71)	0.99 (0.26 – 3.77)
Proton pump inhibitors	34 (44.7)	92 (27.1)	2.17 (1.30 – 3.63) †	2.04 (1.18 – 3.51) †
Vitamins	6 (7.9)	26 (7.7)	1.03 (0.41 – 2.60)	0.84 (0.31 – 2.27)
Mineral supplements	14 (18.4)	31 (9.1)	2.24 (1.13 – 4.46) †	2.51 (1.20 – 5.22) †
Diuretics	20 (26.3)	76 (22.4)	1.23 (0.70 – 2.19)	0.93 (0.48 – 1.78)
Calcium channel blockers	13 (17.1)	44 (13.0)	1.38 (0.70 – 2.72)	1.23 (0.61 – 2.48)
RAS-acting agents	29 (38.2)	81 (23.9)	1.97 (1.16 – 3.33) †	1.63 (0.93 – 2.86)
Corticoids for systemic use	10 (13.2)	38 (11.2)	1.20 (0.57 – 2.53)	1.17 (0.53 – 2.57)

Odds Ratios (OR) and 95 % confidence intervals (95% CI) for resistance to ciprofloxacin in urinary tract infections for drugs prescribed concomittantly with the last fluoroquinolone prescription before culture, both unadjusted and adjusted. ORs were adjusted in for age, sex, kidney function, diabetes, BMI, SES and follow-up time. Proton pump inhibitors were thought to possibly change the pharmacokinetics of fluoroquinolones, while H_2-receptor antagonists were analysed as other major group of acid-lowering drugs. Mineral supplements and vitamins were analysed for their potential interaction with fluoroquinolones in the GI tract. Diuretics and agents acting on the renin-angiotensin system were selected because of their potential influence on the secretion of fluoroquinolones by the kidney, while calcium channel blockers are the other large group of antihypetensive drugs. Corticoids were studied because of their effect on the immune system. † significant with p <0,05 for the adjusted OR.

It was shown that the introduction of fluoroquinolones into broiler chicken production in the USA was associated with an increase in ciprofloxacin-resistant campylobacters in humans.[46] On the other hand, in Australia and Finland, where the use of fluoroquinolones in food animals is concomitant use of levofloxacin and DTCCs has been shown to increase selection of fluoroquinolone-resistant bacteria.[38] Here, we showed that concomitant prescription of calcium supplements and proton pump inhibitors with fluoroquinolones was associated with ciprofloxacin-resistant *E. coli.*

prohibited, only a low prevalence of fluoroquinolone resistance has been found in human samples: in 2010 in *E. coli* only 5.2% and far below 10%, respectively.[47,48] Also, in 99 women with UTIs, an association was shown between more frequent consumption of chicken and MDR *E. coli* in UTIs, and associations have also been found between more frequent consumption of pork and ampicillin- or cephalosporin-resistant *E. coli* in UTIs.[49] To our knowledge, this study is the first to report an association between meat intake and ciprofloxacin-resistant *E. coli* in UTIs.

Previously established risk factors for the development of fluoroquinolone resistance were age and use of fluoroquinolones.[20,24,25] The literature contains conflicting results for kidney function, possibly caused by differences in adjustment for confounding.[21,22] We also showed different results in the different models. The dose of fluoroquinolones is adapted in patients with renal insufficiency, which is a possible explanation, although dose itself was not associated with ciprofloxacin resistance in the group of participants with at least one fluoroquinolone prescription (data not shown). Furthermore, renal insufficiency has been reported as a risk factor for UTIs, while UTIs could progress kidney disease.[50–52] We therefore cannot conclude whether renal insufficiency should be considered as a risk factor for ciprofloxacin resistance. Although we did not confirm an association with diabetes, others have done so.[23] Patients with diabetes have more UTIs and use more antibiotics. We corrected for fluoroquinolone use, possibly explaining this difference. Furthermore, in model 2, sex is borderline significant, while it is not in model 1, probably caused by the fact that men were prescribed more fluoroquinolones than women, for which is not corrected in model 2. Finally, some studies showed associations between the previous use of other antimicrobial drugs, such as nitrofurantoin.[53,54] We studied the previous use of penicillins, trimethoprim derivatives and nitrofuran derivatives, but found no significant associations.

Our study has strengths, but also some potential limitations. The size of the cohort, its real-life population-based character, the length of follow-up, and the prospective and unbiased collecting of information on determinants and outcome are substantial strengths of this study. A limitation may be that dietary data were collected using self-report, which is prone to measurement error. To account for potential systematic error, we adjusted the dietary variables for energy intake.[19] Moreover, the FFQ has been validated in a comparable population and showed adequate ranking of individuals according to their dietary intake.[17,18] Furthermore, we cannot take into account calcium supplements obtained over the counter without prescription and we cannot exclude that individuals who were concomitantly prescribed calcium supplements or proton pump inhibitors and ciprofloxacin were warned not to use both drugs at the same time. However, both would have led to an underestimation of the association. We also cannot exclude the possibility that the urinary tract of some patients was only colonized by *E. coli*. We choose to keep the term UTI, since the urine was sent in by GPs, which, in the Netherlands, is only done when a UTI is suspected and under strict restrictions.[55] A final limitation is the number of statistical tests carried out in this study. However, most of our analyses were hypothesis driven. For those that were not, we preferred to study them one-by-one to see if they made sense and if so, to report them with the advice to replicate instead of using a Bonferroni correction. The risk with Bonferroni correction may be that interesting new genuine associations are missed. Given the multiple comparisons, interpretation of the results should be made with caution.

In conclusion, in an elderly primary care cohort possible modifiable factors for the selection of ciprofloxacin resistance seem to be a high nutritional intake of pork and chicken and concomitantly prescribed calcium supplements and proton pump inhibitors. Modification of antibiotic use in animals or diminishing meat consumption as well as temporarily stopping the prescription of concomitant drugs that could affect bioavailability need further evaluation as prevention strategies for ciprofloxacin resistance.

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Chapter 3.2

Use of Other Antimicrobial Drugs Is Associated with Trimethoprim Resistance in Patients with Urinary Tract Infections caused by *E.coli*.

Marlies Mulder, Annelies Verbon, Jan Lous, Wil Goessens, Bruno Stricker

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Abstract

Objective In recent years, high frequencies of trimethoprim resistance in urinary tract infections (UTIs) caused by *E. coli* are have been reported. Co-resistance to other antimicrobial drugs may play a role in this increase. Therefore, we investigated whether previous use of other antimicrobial drugs was associated with trimethoprim resistance.

Methods We conducted a nested case-control study with urinary cultures with *E. coli* from participants of the Rotterdam Study sent in by general practitioners to the regional laboratory between 1 January 2000 and 1 April 2016. Multivariable logistic regression analysis was performed to study the association between prior prescriptions of several antimicrobial drug groups and trimethoprim resistance using individual participant data.

Results Urinary cultures of 1264 individuals with a UTI caused by *E. coli* were included. When adjusted for previous other antimicrobial drug use, a history of > 3 prescriptions of extended-spectrum penicillins (OR 1.68; 95% CI 1.10–2.55) was significantly associated with trimethoprim resistance of *E. coli* as was the use of > 3 prescriptions of sulfonamides and trimethoprim (OR 2.22; 95% CI 1.51–3.26). The use of > 3 prescriptions of nitrofuran derivatives was associated with a lower frequency of trimethoprim resistance (OR 0.60; 95% CI 0.39–0.92), after adjustment for other antimicrobial drug prescriptions.

Conclusions We found that previous use of extended-spectrum penicillins is associated with trimethoprim resistance. On the contrary, previous nitrofurantoin use was associated with a lower frequency of trimethoprim resistance. Especially in individuals with recurrent UTI, co-resistance should be taken into account and susceptibility testing before starting trimethoprim should be considered.

Introduction

The increase in antimicrobial resistance is becoming a threat to the treatment of infections and is directly associated with the increasing use of antimicrobial drugs.[1] Antimicrobial drugs are frequently prescribed for urinary tract infections (UTIs) by general practitioners (GPs). Trimethoprim has often been prescribed, especially in women with UTIs, and high frequencies of trimethoprim resistance have been reported.[2] Urinary cultures from female students from the USA, positive for *E. coli* of 2005–2007, were in 29.6% of the cases resistant to trimethoprim. In the UK, there was 29% resistance in community UTIs in 2015 and 34% in 2016. In the Netherlands, the resistance rates for trimethoprim in outpatient UTIs caused by *E. coli* increased from 15% in 2000 to 31% in 2010.[3-5] Use of trimethoprim or other antibiotics was shown to be an important risk factor.[6] Co-resistance, the simultaneous resistance of one microbe for two or more antimicrobial drugs, has been suggested to play a role.[4] For example, co-resistance for trimethoprim and amoxicillin means that use of amoxicillin not only selects for amoxicillin-resistant but also for trimethoprim-resistant microorganisms. One of the mechanisms may be that resistance genes are present on the same plasmid.[7]

That co-resistance may be of importance was described in a recent study in the UK, which showed an association between higher prescribing rates of extended-spectrum penicillins (such as amoxicillin) and trimethoprim resistance in *Enterobacteriaceae* causing UTIs. Interestingly, an association with reduced trimethoprim resistance was shown for nitrofurantoin and macrolide use.[8] These associations were at population level and not based on data from individual patients.

In recent years, trimethoprim has been replaced by nitrofurantoin in primary care guidelines in the UK and the Netherlands.[4,5,9,10] Although, trimethoprim resistance rates substantially decreased with the changing guidelines, they remain high with 24% resistance in *E. coli* causing UTIs in primary care patients in the Netherlands in 2017.[11] With the unique opportunity of urinary cultures and antimicrobial drug prescriptions at the individual level from participants of the prospective population-based Rotterdam Study, we were able to study the effects of prescriptions of several antimicrobial drug groups on trimethoprim resistance.

Materials and methods

Source population

We conducted a nested case-control study, using urinary cultures obtained from participants of the Rotterdam Study, a prospective cohort study in older adults in the Ommoord area, Rotterdam, The Netherlands. The Rotterdam Study was described elsewhere.[12] In short, the study started in 1991, when all inhabitants of Ommoord aged ≥ 55 years were invited to participate (78% response rate). New cohorts of inhabitants of ≥ 45 years were included later,

resulting in 14,926 participants in three cohorts. All participants are invited every few years for interviews and examinations. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Study population

The study population included all participants of the Rotterdam Study of whom at least one urinary culture was assessed at the Star-SHL laboratory between January 1, 2000, and April 12, 2016, and which was positive (at least 10^3 cfu/mL) for *E. coli*. The Star-SHL laboratory provides laboratory services for all GPs in the Rotterdam area, including Ommoord. Midstream urine was collected and sent to the Star-SHL laboratory according to the national guidelines of routine care for GPs in The Netherlands. These guidelines recommend culturing only when the patient has clinical signs of a UTI, belongs to a risk group, suffers from a complicated infection, or has complaints that did not disappear despite empiric treatment. When multiple cultures of one individual were sent in, only the first one was used.

Cases and controls

Cases were individuals with a first urinary culture positive for *E. coli* resistant to trimethoprim, whereas controls were individuals with a first urinary culture positive for *E. coli* susceptible to trimethoprim in the study period. Before 2010, disk diffusion was used to determine the susceptibility of the *E. coli* to trimethoprim, whereas after 2010, susceptibility testing was performed with the VITEK 2 system (VITEK AMS; bioMerieux Vitek systems, Inc., Hazelwood, MO, USA). In order to use the same cut-off points, all MIC-values were interpreted according to the CSLI criteria of before 2010. Intermediate susceptibility was considered resistant in the analysis, resulting in a cut-off of > 4 µg/mL. During the period of disk diffusion susceptibility testing (before 2010), the MIC breakpoint of trimethoprim has changed from > 8 to > 4 µg/mL. This may have led to misclassification in this period, but since only 2 out of 586 isolates had a MIC of 4 µg/mL, it can safely be assumed that only a very small proportion of the cultures could have been misclassified. Furthermore, susceptibility of the *E. coli* for amoxicillin (> 8 µg/mL), amoxicillin-clavulanic acid (> 8/4 µg/mL), nitrofurantoin (> 32 µg/mL), and ciprofloxacin (> 4 µg/mL) were determined.

Use of antimicrobial drugs

Computerized records from the pharmacies in the Ommoord district were used to study medication use of the participants. The total number of prescriptions of all antimicrobial drugs between January 1, 1995, and the date of the culture was determined. All generations of cephalosporins were excluded because they were only prescribed to 3 individuals in the study period. This resulted in the following antimicrobial drug groups: ATC-codes J01AA (tetracyclines),

J01CA (extended-spectrum penicillins), J01CR (combinations of penicillins incl. beta-lactamase inhibitors, J01E (sulfonamides and trimethoprim), J01F (macrolides and lincosamides), J01MA (fluoroquinolones), J01XE (nitrofuran derivatives). For each antimicrobial drug group, exposure per individual was categorized according to the number of prescriptions during the study period: 0, 1, 2 or 3, or > 3 prescriptions.

Furthermore, for all groups, the date of the last prescription before culture was obtained, after which the time interval between the antimicrobial drug prescription and urinary culture was calculated. These time intervals were categorized into use 1–3 months, 3–12 months, and > 12 months before urinary culture and compared with no use at all during the study period. Prescriptions in the month before culture were excluded from the analysis, since it could not be excluded that these antimicrobial drugs were prescribed for the UTI that led to the culture.

Confounders

All analyses were adjusted for sex and age. Serum creatinine was used to calculate the glomerular filtration rate (GFR), according to the CKD-EPI equation, using the value closest to culture.[13] Diabetes mellitus was defined as use of anti-diabetic medication at the moment of culture. Socioeconomic status (SES) was scored at baseline according to the UNESCO criteria. To account for potential bias associated with missing data, missing values on kidney function (18.4%) and SES (1.3%) were imputed using multiple imputation (N = 20 imputations) in SPSS using the default settings.[14] All available variables (sex, age, diabetes, the use of the different antimicrobial drugs, and the different resistance rates) were used as predictor variables. Furthermore, follow-up time was calculated as the time between start of the study (1 January 2000) and urinary culture and used as a potential confounder in the models.

Analysis and statistical methods

Binary logistic regression was used to study the association between categorized use of antimicrobial drugs per group as defined above and trimethoprim resistance (no/yes). For each antimicrobial drug, we first calculated univariable ORs. Second, we performed a multivariable analysis for each antimicrobial drug group in which the OR was adjusted for the potential confounders age, sex, GFR, SES, diabetes, and follow-up time (model 1). Third, we performed an overall analysis of all antimicrobial drug groups in which the ORs of the antimicrobial drug groups were adjusted for all confounders and adjusted for the use of all other studied antimicrobial drug groups (model 2). For example, in model 2, the use of sulfonamides and trimethoprim was adjusted for age, sex, GFR, SES, diabetes, follow-up time, use of tetracylines, use of extended-spectrum penicillins, use of combinations of penicillins, incl. enzyme inhibitors, use of macrolides and lincosamides, use of fluoroquinolones, and use of nitrofurantoin during the study period. Furthermore, Spearman's correlation coefficients were calculated for the use of all antimicrobial drug groups.

$p < 0.05$ was considered to be statistically significant. All statistical analyses were performed with IBM SPSS Statistics 24.

Results

The study population consisted of 1264 individuals with urinary cultures positive for *E. coli* of whom 1011 (80.0%) were women, and with a median age of 75 years. The percentage resistance to trimethoprim of all *E. coli* isolates was 31.1%. Resistance percentages to other antimicrobials in trimethoprim-resistant *E. coli* were higher than in trimethoprim-sensitive *E. coli* isolates: 86.3% versus 26.5% were co-resistant to amoxicillin, 26.2% versus 9.2% to amoxicillin-clavulanic acid, 22.4% versus 4.7% to ciprofloxacin, and 13.0% versus 4.2% to nitrofurantoin (**Table 1**). Of all included individuals, 893 (70.6%) were prescribed at least one beta-lactam antimicrobial drug during the study period, 745 (58.9%) a sulfonamide and trimethoprim, 466 (39.9%) a fluoroquinolone, and 603 (47.7%) nitrofurantoin (**Table 1**).

Although resistance to all other antimicrobial drugs was higher in the trimethoprim-resistant *E. coli* isolates than in the trimethoprim-sensitive *E. coli* isolates, the overall use of antimicrobial drugs was not significantly higher in individuals with trimethoprim-resistant *E. coli* isolates. We also studied the correlations between the use of different antibiotic classes, which were all negligible to low and none of them was negative. For example, participants who had received a higher number of sulphonamides and trimethoprim prescriptions also had been prescribed a higher number of prescriptions of all other antimicrobial drug groups, but these correlations were low (**Table 2**).

Several antimicrobial drug classes, such as tetracyclines and fluoroquinolones, seemed to be associated with trimethoprim resistance in model 1, but this effect disappeared in model 2, in which antimicrobial drug use of one class was adjusted for potential confounding by use of other antimicrobial drug groups. In model 2, we showed that > 3 prescriptions of extended-spectrum penicillins were associated with trimethoprim resistance (OR 1.68; 95% CI 1.10–2.55), whereas combinations of penicillins incl. enzyme inhibitors were not. Also, > 3 prescriptions of a sulfonamide and trimethoprim were significantly associated with trimethoprim resistance (OR 2.22; 95% CI 1.51–3.26). In contrast, > 3 nitrofurantoin prescriptions were significantly associated with a lower frequency of resistance to trimethoprim (OR 0.60; 95% CI 0.39–0.92) (**Table 3**).

The time period since the last prescription of extended-spectrum penicillins (1–3 months; OR 2.86; 95% CI 1.29–6.34) was associated with trimethoprim resistance in model 2. This association was not seen for combinations of penicillins and enzyme inhibitors. Furthermore, an association

Table 1. General characteristics of study population

	All participants (n = 1264)	**Participants with trimethoprim-resistant *E.coli* in UTI (n = 393)**
Age (years), median (IQR)	75.3 (66.5 – 83.3)	77.2 (67.6 – 85.1)
Women, n (%)	1011 (80.0)	323 (82.2)
Kidney function (GFR), median (IQR)	80.3 (69.8 – 90.1)	79.0 (68.8 – 89.2)
Diabetes, n (%)	170 (13.4)	58 (14.8)
Trimethoprim resistance, n (%)	393 (31.1)	393 (100)
Amoxicillin resistance, n (%)	570 (45.1)	339 (86.3)
Amoxicillin-clavulanic acid, n (%)	183 (14.5)	103 (26.2)
Ciprofloxacin resistance, n (%)	129 (10.2)	88 (22.4)
Nitrofurantoin resistance, n (%)	88 (7.0)	51 (13.0)
Previous use of sulfonamides and trimethoprim, n (%)	745 (58.9)	264 (67.2)
Previous use of tetracyclines, n (%)	759 (60.0)	241 (61.3)
Previous use of extended-spectrum penicillins, n (%)	690 (54.6)	218 (55.4)
Previous use of combinations of penicillins, incl. enzyme inhibitors, n (%)	602 (47.6)	192 (48.9)
Previous use of macrolides and lincosamides, n (%)	568 (44.9)	184 (46.8)
Previous use of fluoroquinolones, n (%)	466 (39.9)	176 (44.8)
Previous use of nitrofurantoin, n (%)	603 (47.7)	188 (47.8)

General characteristics of the study population. The first column shows the characteristics for all participants, whereas the second column shows the characteristics only of participants who had a urinary tract infection caused by a trimethoprim-resistance *E.coli*.

was demonstrated between the use of sulfonamides and trimethoprim in the 1–3 months before culture (OR 2.22; 95% CI 1.27–3.87) and trimethoprim resistance. Although there was no association with the use of fluoroquinolones 1–3 months before culture, there was one for 3–12 months (OR 2.28; 95% CI 1.33–3.19). No association was found between the use of nitrofurantoin and trimethoprim resistance in this model 2 using the time intervals (**Table 4**). However, when adjusting the timing of the last nitrofurantoin prescription for the number of prescriptions of the other antimicrobial drug groups instead of the timing of the last prescription of these groups, nitrofurantoin was associated with less trimethoprim resistance (1–3 months: OR 0.50; 95% CI 0.27–0.90).

Discussion

In this study, we showed that trimethoprim use and extended-spectrum penicillins use (such as amoxicillin) were significantly associated with trimethoprim resistance in *E. coli* causing UTIs,

Table 2: Spearman's rank correlation coefficients for the use of antimicrobial drug groups

	SFX-TMP	TCN	Ex-PCN	Comb-PCN-EI	M-L	FQ	NFT
SFX-TMP	-	0.19	0.09	0.15	0.14	0.40	0.39
TCN	0.19	-	0.30	0.25	0.38	0.21	0.19
Ex-PCN	0.09	0.30	-	0.30	0.21	0.10	0.11
Comb-PCN-EI	0.15	0.25	0.30	-	0.21	0.25	0.13
M-L	0.13	0.38	0.21	0.21	-	0.17	0.17
FQ	0.40	0.21	0.10	0.25	0.17	-	0.29
NFT	0.39	0.19	0.11	0.13	0.17	0.29	-

Spearman's rank correlation coefficients for the correlations between the use of different antimicrobial drug groups. The Spearman's rank correlation coefficient is used because of skewed distribution of the variables. The strength of the relation can be between -1 (perfect negative correlation) to 1 (perfect positive correlation) with 0 meaning no correlation present. As a rule of thumb, the size of the strength of the correlation can be interpreted as follows: 0.9 to 1.0 (-0.9 to -1.0) very high correlation; 0.70 to 0.90 (-0.70 to -0.90) high correlation; 0.50 to 0.70 (-0.50 to -0.70) moderate correlation; 0.30 to 0.50 (-0.30 to -0.50) low correlation and 0.0 to 0.30 (0.0 to -0.30) negligible correlation.[53] SFX-TMP = Sulfonamides and trimethoprim; TCN = Tetracyclines; Ex-PCN = Extended-spectrum penicillins; Comb-PCN-EI = Combinations of penicillins with enzyme inhibitors; M-L = Macrolides and lincosamides; FQ = Fluoroquinolones; NFT = Nitrofurantoin.

both for the number of prescriptions (> 3 prescriptions) as well as for the time interval between the last prescription and culture (prescription 1–3 months before culture). Additionally, the use of > 3 prescriptions of nitrofurantoin was associated with a lower frequency of trimethoprim resistance on a patient level. Both these results confirm at an individual patient level the findings from the association study at population level, mentioned in the "Introduction" section.[5]

Nitrofurantoin use, as assessed by prescriptions, was associated with a lower frequency of trimethoprim resistance, but only after adjustment for the number of other antimicrobial drug prescriptions, including trimethoprim. This suggests that nitrofurantoin use is inversely associated with trimethoprim resistance in individuals with a high frequency of antimicrobial drug use, possibly due to recurrent UTIs. This result was not found in the model that studied the timing of the last prescription, possibly because participants who were prescribed nitrofurantoin shortly before the urine culture were not or less frequently prescribed another antimicrobial drug for a UTI episode. Co-resistance to trimethoprim and nitrofurantoin was low (13%), but higher than overall resistance to nitrofurantoin (7%) and there was a low but positive correlation between sulfonamides use and trimethoprim or nitrofurantoin use. Thus, lower trimethoprim

resistance might not to be caused by a decreased frequency of trimethoprim use, but it may be hypothesized that trimethoprim-resistant *E. coli* are eradicated by nitrofurantoin.

Despite the high frequency of use, resistance to nitrofurantoin remains rather low in contrast to other antimicrobial drugs.[11] This might be explained by the observation that *E. coli* mutants resistant to nitrofurantoin were less able to multiply, which is a disadvantage compared with nitrofurantoin-sensitive *E. coli*.[16] Furthermore, the nfsA and nfsB, genes, which play a role in nitrofurantoin resistance are chromosomal and not plasmid-mediated, diminishing the chance of transfer of these genes to other bacteria. However, recently, the plasmid-mediated *oqxAB* has also been shown to play a role in nitrofurantoin resistance.[17] These characteristics of nitrofurantoin make it an antimicrobial drug of high interest in this era of antimicrobial resistance.

The association between sulfonamides and trimethoprim use and trimethoprim resistance was expected and has been described before.[6,18] We also confirm at the patient level the association between extended-spectrum penicillins use, such as amoxicillin, and trimethoprim resistance.[8] Furthermore, the correlation between sulfonamides and trimethoprim use and extended-spectrum penicillins use is low. Moreover, since we adjusted for trimethoprim use in the model, it seems unlikely that the association between amoxicillin use and trimethoprim resistance is the result of a combination of a high number of prescriptions of amoxicillin and trimethoprim. Our data suggest that co-resistance plays an important role, especially in individuals with high antimicrobial drug use.

The association between fluoroquinolones use and trimethoprim resistance remains unclear. In our analyses, the association between fluoroquinolone use and trimethoprim resistance disappeared after adjustment for other antimicrobial drug groups. In the time interval model, the association with the last prescription 3–12 months before culture could not be confirmed in other time periods. The association between fluoroquinolones use and trimethoprim resistance should therefore be investigated further. Also, we did not find any associations with macrolides and lincosamides use, although Pouwels et al. did find a protective effect of macrolide use on the resistance to trimethoprim.[8] This is possibly explained by differences in prescribing patterns between the Netherlands and UK.

A strength of this study is that we confirmed the results of an earlier association study with aggregated general practice data[5] using a nested case-control design with individual patient data. On the contrary, a limitation of the study is the fact that we use filled prescription data for antimicrobial drugs but do not know whether patients were adherent to pharmacotherapy. However, since individuals with infections visit their GP because of complaints and antibiotics are usually seen by patients as safe and effective, it is reasonable to assume that patients actually

Table 3. Associations between previous use of antimicrobial drug groups and trimethoprim resistance.

Sulfonamides and trimethoprim	**No use**	**1 prescription**	**2 or 3 prescriptions**	**>3 prescriptions**
Number of cases (%)	129 (32.8)	80 (20.4)	75 (19.1)	109 (27.7)
Number of controls (%)	390 (44.8)	178 (20.4)	167 (19.2)	136 (15.6)
OR (95% CI) univariable	ref	1.36 (0.98 – 1.89)	1.36 (0.97 – 1.90)	2.42 (1.76 – 3.34)*
OR (95% CI) in model 1	ref	1.40 (1.00 – 1.95)*	1.32 (0.94 – 1.86)	2.28 (1.64 – 3.17)*
OR (95% CI) in model 2	ref	1.45 (1.03 – 2.05)*	1.32 (0.92 – 1.89)	2.22 (1.51 – 3.26)*
Tetracyclines				
Number of cases (%)	152 (38.7)	84 (21.4)	61 (15.5)	96 (24.4)
Number of controls (%)	353 (40.5)	178 (20.4)	178 (20.4)	162 (18.6)
OR (95% CI) univariable	ref	1.10 (0.79 – 1.51)	0.80 (0.56 – 1.13)	1.38 (1.00 – 1.89)*
OR (95% CI) in model 1	ref	1.10 (0.79 – 1.52)	0.84 (0.59 – 1.19)	1.43 (1.03 – 1.97)*
OR (95% CI) in model 2	ref	0.98 (0.69 – 1.37)	0.72 (0.50 – 1.05)	1.11 (0.76 – 1.61)
Extended-spectrum penicillins				
Number of cases (%)	175 (44.5)	83 (21.1)	72 (18.3)	63 (16.0)
Number of controls (%)	399 (45.8)	216 (24.8)	164 (18.8)	92 (10.6)
OR (95% CI) univariable	ref	0.88 (0.64 – 1.19)	1.00 (0.72 – 1.39)	1.56 (1.08 – 2.25)*
OR (95% CI) in model 1	ref	0.93 (0.68 – 1.27)	1.07 (0.77 – 1.50)	1.80 (1.23 – 2.63)*
OR (95% CI) in model 2	ref	0.93 (0.67 – 1.28)	1.04 (0.73 – 1.48)	1.68 (1.10 – 2.55)*
Combinations of penicillins, incl. enzyme inhibitors				
Number of cases (%)	201 (51.1)	84 (21.4)	62 (15.8)	46 (11.7)
Number of controls (%)	461 (52.9)	186 (21.4)	142 (16.3)	82 (9.4)
OR (95% CI) univariable	ref	1.03 (0.76 – 1.41)	1.00 (0.71 – 1.41)	1.29 (0.87 – 1.91)
OR (95% CI) in model 1	ref	1.06 (0.78 – 1.45)	1.09 (0.77 – 1.54)	1.39 (0.92 – 2.08)
OR (95% CI) in model 2	ref	0.98 (0.71 – 1.36)	0.91 (0.63 – 1.32)	0.98 (0.62 – 1.55)

Macrolides and lincosamides				
Number of cases (%)	209 (53.2)	88 (22.4)	55 (14.0)	41 (10.4)
Number of controls (%)	487 (55.9)	168 (19.3)	132 (15.2)	84 (9.6)
OR (95% CI) univariable	ref	1.22 (0.90 – 1.66)	0.97 (0.68 – 1.38)	1.14 (0.76 – 1.71)
OR (95% CI) in model 1	ref	1.31 (0.96 – 1.79)	1.06 (0.74 – 1.53)	1.27 (0.83 – 1.91)
OR (95% CI) in model 2	ref	1.21 (0.87 – 1.67)	0.90 (0.61 – 1.32)	0.97 (0.61 – 1.54)
Fluoroquinolones				
Number of cases (%)	217 (55.2)	63 (16.0)	58 (14.8)	55 (14.0)
Number of controls (%)	581 (66.7)	118 (13.5)	95 (10.9)	77 (8.8)
OR (95% CI) univariable	ref	1.43 (1.01 – 2.02)*	1.64 (1.14 – 2.35)*	1.91 (1.31 – 2.80)*
OR (95% CI) in model 1	ref	1.45 (1.02 – 2.06)*	1.59 (1.10 – 2.29)*	1.89 (1.28 – 2.79)*
OR (95% CI) in model 2	ref	1.33 (0.92 – 1.93)	1.43 (0.96 – 2.13)	1.51 (0.96 – 2.37)
Nitrofurantoin				
Number of cases (%)	205 (52.2)	64 (16.3)	69 (17.6)	55 (14.0)
Number of controls (%)	456 (52.4)	163 (18.7)	130 (14.9)	122 (14.0)
OR (95% CI) univariable	ref	0.87 (0.63 – 1.22)	1.18 (0.84 – 1.65)	1.00 (0.70 – 1.44)
OR (95% CI) in model 1	ref	0.85 (0.60 – 1.19)	1.11 (0.79 – 1.57)	0.99 (0.68 – 1.44)
OR (95% CI) in model 2	ref	0.72 (0.51 – 1.04)	0.86 (0.59 – 1.27)	0.60 (0.39 – 0.92)*

Associations between the previously prescribed number (1, 2 or 3, >3 compared to none) of prescriptions of antimicrobial agents for individuals with a UTI caused by an *E.coli* resistant to trimethoprim (cases) compared to individuals with a UTI caused by an *E.coli* susceptible to trimethoprim (controls). For each antimicrobial drug group, it shows the univariable OR, the OR adjusted for the possible confounders sex, age, diabetes, GFR, SES and follow-up time (model 1) and the OR adjusted for sex, age, diabetes, GFR, SES and follow-up time, and the use of other antimicrobial drug group prescriptions (model 2). *significant with $p<0.05$.

Table 4. Associations between the timing of the last prescription of several antimicrobial drug groups and trimethoprim resistance.

Sulfonamides and trimethoprim	**no use**	**>12 months**	**3-12 months**	**1-3 months**
Number of cases (%)	110 (28.0)	101 (25.7)	37 (9.4)	25 (6.4)
Number of controls (%)	377 (43.3)	327 (37.5)	73 (8.4)	39 (4.5)
OR (95% CI) univariable	ref	1.06 (0.78 – 1.44)	1.74 (1.11 – 2.72)*	2.20 (1.27 – 3.79)*
OR (95% CI) in model 1	ref	1.04 (0.76 – 1.42)	1.71 (1.08 – 2.70)*	2.23 (1.28 – 3.88)*
OR (95% CI) in model 2	ref	1.00 (0.72 – 1.39)	1.66 (1.00 – 2.77)*	2.22 (1.27 – 3.87)*
OR (95% CI) adjusted for number	ref	1.01 (0.72 – 1.42)	1.55 (0.95 – 2.52)	2.24 (1.25 – 4.02)*
Tetracyclines				
Number of cases (%)	152 (38.7)	197 (50.1)	25 (6.4)	12 (3.1)
Number of controls (%)	352 (40.4)	437 (50.2)	62 (7.1)	16 (1.8)
OR (95% CI) univariable	ref	1.04 (0.81 – 1.35)	0.93 (0.57 – 1.54)	1.74 (0.80 – 3.76)
OR (95% CI) in model 1	ref	1.07 (0.82 – 1.39)	0.92 (0.56 – 1.52)	1.80 (0.83 – 3.93)
OR (95% CI) in model 2	ref	0.90 (0.66 – 1.21)	0.75 (0.43 – 1.32)	1.50 (0.64 – 3.53)
OR (95% CI) adjusted for number	ref	0.89 (0.66 – 1.18)	0.79 (0.48 – 1.29)	1.52 (0.68 – 3.39)
Extended-spectrum penicillins				
Number of cases (%)	174 (44.3)	166 (42.2)	20 (5.1)	16 (4.1)
Number of controls (%)	393 (45.1)	379 (43.5)	52 (6.0)	15 (1.7)
OR (95% CI) univariable	ref	0.99 (0.77 – 1.28)	0.87 (0.50 – 1.50)	2.41 (1.17 – 4.98)*
OR (95% CI) in model 1	ref	1.06 (0.81 – 1.37)	0.97 (0.56 – 1.69)	2.74 (1.31 – 5.73)*
OR (95% CI) in model 2	ref	1.01 (0.75 – 1.36)	0.95 (0.52 – 1.74)	2.86 (1.29 – 6.34)*
OR (95% CI) adjusted for number	ref	1.00 (0.76 – 1.32)	0.87 (0.49 – 1.56)	2.91 (1.36 – 6.23)*
Combinations of penicillins, incl. enzyme inhibitors				
Number of cases (%)	186 (47.3)	128 (32.6)	31 (7.9)	16 (4.1)
Number of controls (%)	432 (49.6)	277 (31.8)	57 (6.5)	28 (3.2)
OR (95% CI) univariable	ref	1.07 (0.82 – 1.41)	1.26 (0.79 – 2.02)	1.33 (0.70 – 2.51)
OR (95% CI) in model 1	ref	1.16 (0.88 – 1.53)	1.24 (0.77 – 1.99)	1.31 (0.68 – 2.50)
OR (95% CI) in model 2	ref	1.11 (0.81 – 1.52)	0.96 (0.73 – 1.28)	1.26 (0.62 – 2.57)
OR (95% CI) adjusted for number	ref	1.00 (0.75 – 1.35)	1.00 (0.62 – 1.61)	1.07 (0.54 – 2.12)

Macrolides and lincosamides				
Number of cases (%)	208 (52.9)	148 (37.7)	22 (5.6)	9 (2.3)
Number of controls (%)	484 (55.6)	311 (35.7)	50 (5.7)	15 (1.7)
OR (95% CI) univariable	ref	1.11 (0.86 – 1.43)	1.02 (0.60 – 1.73)	1.40 (0.60 – 3.24)
OR (95% CI) in model 1	ref	1.21 (0.93 – 1.58)	1.13 (0.66 – 1.92)	1.43 (0.60 – 3.37)
OR (95% CI) in model 2	ref	1.07 (0.80 – 1.42)	0.90 (0.51 – 1.60)	1.35 (0.55 – 3.29)
OR (95% CI) adjusted for number	ref	1.04 (0.77 – 1.40	0.91 (0.50 – 1.64)	1.30 (0.50 – 3.26)
Fluoroquinolones				
Number of cases (%)	209 (53.2)	97 (24.7)	33 (8.4)	19 (4.8)
Number of controls (%)	559 (64.2)	193 (22.2)	37 (4.2)	25 (2.9)
OR (95% CI) univariable	ref	1.34 (1.01 – 1.80)*	2.39 (1.45 – 3.92)*	2.03 (1.09 – 3.77)*
OR (95% CI) in model 1	ref	1.34 (0.99 – 1.80)	2.50 (1.51 – 4.15)*	1.86 (0.99 – 3.48)
OR (95% CI) in model 2	ref	1.19 (0.84 – 1.67)	2.02 (1.14 – 3.65)*	1.66 (0.83 – 3.34)
OR (95% CI) adjusted for number	ref	1.13 (0.81 – 1.58)	2.28 (1.33 – 3.19)*	1.59 (0.82 – 3.07)
Nitrofurantoin				
Number of cases (%)	180 (45.8)	91 (23.2)	39 (9.9)	17 (4.3)
Number of controls (%)	386 (44.3)	183 (21.0)	64 (7.3)	57 (6.5)
OR (95% CI) univariable	ref	1.07 (0.78 – 1.45)	1.31 (0.85 – 2.02)	0.64 (0.36 – 1.13)
OR (95% CI) in model 1	ref	0.99 (0.72 – 1.37)	1.27 (0.84 – 1.92)	0.64 (0.36 – 1.12)
OR (95% CI) in model 2	ref	0.82 (0.57 – 1.19)	0.91 (0.57 – 1.47)	0.54 (0.29 – 1.01)
OR (95% CI) adjusted for number	ref	0.76 (0.54 – 1.08)	0.97 (0.61 – 1.53)	0.50 (0.27 – 0.90)*

Associations between the timing (1-3 months, 3-12 months, >12 months before culture compared to no use) of the last prescription of antimicrobial agents for individuals with a UTI caused by an *E.coli* resistant to trimethoprim (cases) compared to individuals with a UTI caused by an *E.coli* susceptible for trimethoprim (controls). For each antimicrobial drug group, it shows the univariable OR, the OR adjusted for the possible confounders sex, age, diabetes, GFR, SES and follow-up time (model 1) and the OR adjusted for the sex, age, diabetes, GFR, SES and follow-up time, and antimicrobial drug group prescriptions of the other antimicrobial drug groups (model 2). *significant with $p<0.05$.

have taken the drugs. Furthermore, our results could be influenced by residual confounding. For example, it cannot be excluded that there are differences between GPs in prescribing antimicrobial drugs and in sending cultures, although there is a national guideline that give recommendations for diagnostics and treatment of UTIs by GPs.[10] Finally, the design of the study did not allow us to confirm our results by studying the genetic associations of resistance genes in these *E. coli* isolates.

In conclusion, in individual patients, the use of extended-spectrum penicillins, such as amoxicillin, is associated with trimethoprim resistance, possibly via selection by co-resistance. Importantly, use of nitrofurantoin is associated with lower trimethoprim resistance. This indicates that co-resistance could be important to consider when prescribing antimicrobial drugs and that resistance testing is important in individuals with recurrent UTIs.

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Chapter 3.3

Diet as a Risk Factor for Antimicrobial Resistance in Community-Acquired Urinary Tract Infections in a Middle-Aged and Elderly Population: a Case-Control Study.

Marlies Mulder, Jessica Kiefte - de Jong, Wil Goessens, Herman de Visser, Arfan Ikram, Annelies Verbon, Bruno Stricker

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Abstract

Objective There is an ongoing debate as to what extent antimicrobial resistance (AMR) can be transmitted from animals to humans via the consumption of animal products. Because epidemiological data on the role of diet in AMR in humans are lacking, we investigated this association between diet and AMR for different antimicrobial drugs in *Escherichia coli* (E. coli) in urinary tract infections (UTIs).

Methods Susceptibility of *E. coli* in urinary cultures and information on diet (with food frequency questionnaires) were obtained from participants of the Rotterdam study, a population-based prospective cohort study. The association between intake of several food groups (meat, seafood, eggs, dairy products, crops) and resistance of *E. coli* to several antimicrobial drugs (amoxicillin, amoxicillin-clavulanic acid, trimethoprim, sulfamethoxazole-trimethoprim, first-generation cephalosporins, cefotaxime, nitrofurantoin, norfloxacin) was studied.

Results Urinary cultures with *E. coli* were obtained from 612 individuals, of whom 481 (78.6%) were women. Resistance rates varied from 246/611 (40.3%) for amoxicillin and 167/612 (27.3%) for trimethoprim to only 29/612 (4.7%) for nitrofurantoin and 16/462 (3.5%) for cefotaxime. A higher intake of chicken was associated with cefotaxime resistance (OR 2.18; 95% CI 1.05–4.51 per tertile increase); a higher intake of pork was associated with norfloxacin resistance (OR 1.42; 95% CI 1.04–1.95 per quartile increase). In contrast, a higher intake of cheese was associated with lower AMR to amoxicillin (OR 0.84; 95% CI 0.72–0.99 per quartile increase) and amoxicillin-clavulanic acid (OR 0.67; 95% CI 0.53–0.86 per quartile increase).

Conclusions These findings support the hypothesis that diet may play a role in the AMR of *E. coli* in UTIs.

Introduction

Increasing numbers of bacteria causing common infections, such as urinary tract infections (UTIs), are resistant to antimicrobial drugs. In recent decades, much attention has been given to the possible transmission of resistant pathogens or genes from animals to humans, either through direct contact or via ingestion of food, such as meat or crops contaminated through water or soil.[1] Antimicrobials are prescribed in the pig, cattle and chicken industries, and also in the farmed seafood industry.[2] A recent study estimated a global increase of antimicrobials in food animals by two-thirds in the period 2010–2030.[3]

It has been shown that the most prevalent resistance genes are those encoding resistance to antimicrobials that are also used in animals.[4] Furthermore, epidemiological studies in UTIs have suggested associations between high intake of pork and chicken and ciprofloxacin resistance in *Escherichia coli* (*E. coli*),[5] between pork consumption and ampicillin-resistant or third-generation cephalosporin-resistant *E. coli,* and between chicken consumption and ampicillin-resistant *E. coli*.[6] In addition, several studies showed similarities between extended-spectrum β-lactamase genes from *E. coli* in broilers, retail chicken and clinical isolates from humans.[7-9] The debate, however, is ongoing, because more recent studies could not confirm these findings.[10]

It is necessary to assess risk factors for antimicrobial resistance to safeguard the use of antimicrobial therapy in humans in the next decades. This study aims to generate a hypothesis and investigated the association between the intake of several food groups (meat, seafood, eggs, dairy products and crops) and antimicrobial resistance of *E. coli* in UTIs.

Methods

Source population

This retrospective case–control analysis was embedded in The Rotterdam Study, a prospective cohort study in the Ommoord district in Rotterdam, the Netherlands, designed to investigate determinants of disease occurrence and progression. The study started in 1990 and includes 14 926 participants of ≥45 years, of whom 8823 (59.1%) are women and 6103 (40.9%) are men. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study).[11] All participants provided written informed consent.

Study population

The study population consisted of all individuals of the Rotterdam Study with at least one positive urinary culture (at least 10^3 CFU/mL) with *E. coli* between 1 January 2000 and 12 April

2016 received by the Star Medisch Diagnostisch Centrum (which provides laboratory services for general practitioners in Rotterdam) and who completed the food frequency questionnaire (FFQ). Participants with an unusual energy intake of <500 or >5000 kcal/day were excluded (**Figure.1**). Urinary cultures were collected as part of routine clinical care and submitted when individuals had clinical signs suggesting a UTI. In case of multiple cultures, only the first culture was used in the analyses. Part of these urinary cultures have also been used in a study investigating the association between diet and resistance to ciprofloxacin.[5]

Cases and controls

Cases were all individuals of the study population with a positive culture with *E. coli* resistant to the antimicrobial drug of interest, whereas controls were all individuals with a susceptible *E. coli* in their culture. For each *E. coli*, the susceptibility for several antimicrobial drugs was determined (**Figure.1**). Consequently, the same cultures were used in multiple analyses, and individuals who were case in one analysis could be control in another one. The antimicrobials investigated were amoxicillin, amoxicillin-clavulanic acid, trimethoprim, sulfamethoxazole-trimethoprim, first-generation cephalosporins (cefalotin or cefazolin), cefotaxime, nitrofurantoin and norfloxacin. The susceptibility of the *E. coli* to these antimicrobial drugs was determined by disc diffusion before 2010 and from 2010 with the VITEK 2 system (VITEK AMS; bioMérieux Vitek systems, Inc., Hazelwood, MO, USA). All MIC values were interpreted according to the CLSI criteria from before 2010 in order to use the same cut-off for all cultured bacteria in determining a pathogen to be resistant. Applied MIC (disc diffusion) breakpoints were: amoxicillin >8 mg/L (<17 mm), amoxicillin-clavulanic acid >8/4 mg/L (<18 mm), trimethoprim >4 mg/L (<16 mm), sulfamethoxazole-trimethoprim >2/38 mg/L (<16 mm), first-generation cephalosporins >8 mg/L (<18 mm), cefotaxime >8 mg/L (<23 mm), nitrofurantoin >32 mg/L (<17 mm), norfloxacin >4 mg/L (<17 mm). The MIC breakpoint of trimethoprim has changed during the period with no registered MIC values from >8 mg/L to >4 mg/L, meaning that this could have led to a misclassification in this period. Of the registered MIC values for trimethoprim, however, only one out of 320 isolates (0.3%) had a MIC of 4 mg/L, so it is assumed that only a very small proportion of cultures before 2010 could have been misclassified as susceptible.

A sensitivity analysis was performed for all *Enterobacteriaceae* isolates, using every first culture with an *Enterobacteriaceae*. Species that were intrinsically resistant to the antimicrobial were excluded.

Diet

The intake of several food groups was determined, adjusted using the residual method and categorized into quartiles as also performed before (because of the small number of cases for

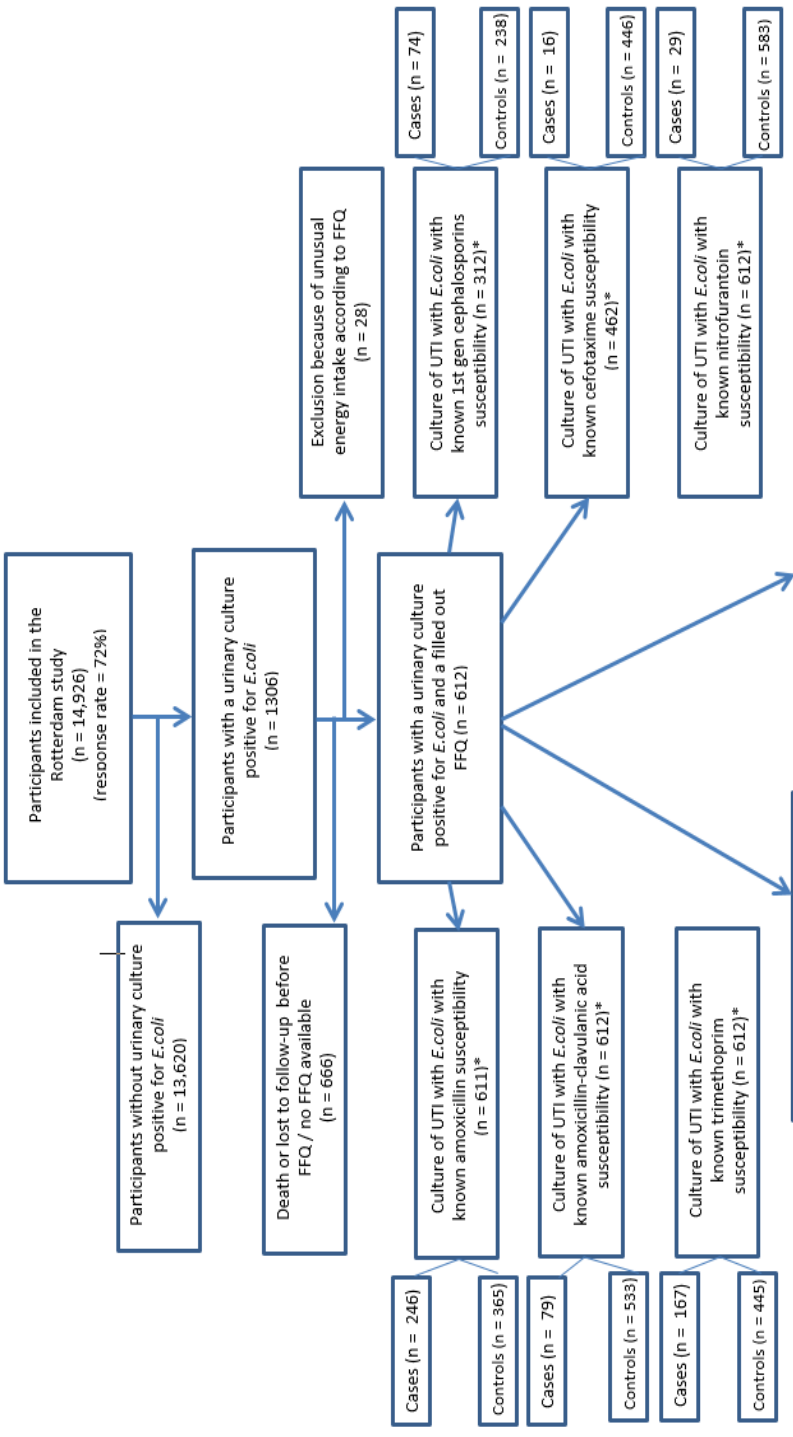

Figure 1: Flowchart of inclusion of participants in the study population.
*The same cultures were used in several analyses to study different antimicrobial drug groups

cefotaxime, categorization was performed in tertiles).[5] Habitual food intake of participants of The Rotterdam Study was determined with an extensive semi-quantitative FFQ, which was developed on the basis of an existing validated FFQ for Dutch adults.[12,13] Total energy intake was calculated using the Dutch Food Composition Tables of 2006 and 2011. Potential measurement error and confounding were accounted for by adjusting dietary intake for total energy intake and using the residual—resulting in dietary intake fixed to the median energy intake of the population (**Appendix S1**).[14] The following food groups were included: beef, chicken, pork, offal, fish, shellfish and crustaceans, animal milk products, eggs, cheese, yoghurt and cottage cheese, fruits, vegetables, and potatoes.

Confounders

Characteristics such as sex, age (at culturing), and body mass index (at time of FFQ) were routinely collected. Serum creatinine was determined and used to calculate the glomerular filtration rate, according to the CKD-EPI equation.[15] Diabetes mellitus was defined as use of anti-diabetic medication. Socio-economic status was scored according to the UNESCO criteria. To account for potential bias associated with missing data, missing values on kidney function (8.3%-9.2%), BMI (3.9%-4.6%) and socio-economic status (1.0%-1.7%) were imputed using multiple imputation (n = 10 imputations).[16] The total number of antimicrobial prescriptions during the period preceding the culture (since 1 January 1995) of the antimicrobial group of interest was obtained from pharmacy records and this variable was used as a confounder in the model. Furthermore, the follow-up time (the time between start of study and urinary culture) and the time between completing FFQ and urinary culture were calculated and added to the multivariate analysis. The multivariate models were also adjusted for the Dutch Healthy Diet index. This variable is a measure for the overall healthy diet pattern assessed by adherence to the Dutch Guidelines for a Healthy Diet [17].[17] When vegetables or fish were included in the model, these components were subtracted from the Dutch Healthy Diet index to prevent over-adjustment.

Analysis and statistical methods

For each antimicrobial drug resistance, an association model was developed to study the dietary intake that best predicts antimicrobial resistance. First, all categorized food groups were univariately tested in the model with absence or presence of antimicrobial resistance (of each antimicrobial drug separately) as an outcome. All categorized food groups with $p < 0.2$ were included in the association model, which also included the previously described potential confounders: sex, age, number of previously prescribed antimicrobial drugs (of same group as antimicrobial drug of interest), kidney function, body mass index, socio-economic status, Dutch Healthy Diet index, follow-up time and time between FFQ and culture. Next, backward selection with $p < 0.1$ as end point (only for the food groups) was conducted to determine the final model. Spearman's correlation coefficients for the food groups were calculated. To prevent co-linearity, food groups with a correlation >0.7 were not added to the same model. After backward selection, additional adjustment was performed for food groups that had a mutual correlation

of >0.2. The Hosmer–Lemeshow test was performed to test the goodness-of-fit of the model; p <0.05 was considered to be statistically significant. All statistical analyses were performed with IBM SPSS Statistics 21.

Results

Of all participants in the Rotterdam Study, 612 had a urinary culture with *E. coli* and completed an FFQ. The median age was 72 years (interquartile range (IQR) 64–79 years) and 481 (78.6%) were women (**Table 1**). The median time between culturing and completing the FFQ was 3.5 years (IQR 1.6–5.6 years). After selecting the food groups with a cut-off of p >0.2 (**Table S1**), an association model was made using backward selection. Most of the correlation coefficients between the different food groups were low and none of them was >0.7. (**Table S2**).

A higher intake of pork was associated with norfloxacin resistance (OR 1.42; 95% CI 1.04–1.95 per quartile increase). Furthermore, a higher intake of chicken was associated with cefotaxime resistance (OR 2.18; 95% CI 1.05–4.51 per quartile increase). In contrast, a higher intake of cheese was associated with lower prevalence of resistance to amoxicillin and amoxicillin-clavulanic acid (OR 0.84; 95% CI 0.72–0.99 and OR 0.67; 95% CI 0.53–0.86 per quartile increase). A higher intake of potatoes was inversely associated with amoxicillin resistance in the highest versus the lowest quartile (OR 0.53; 95% CI 0.32–0.87) (**Table 2**).

In a model that was adjusted for all meat types, the associations of cheese and vegetables did not substantially change.

To achieve more power, sensitivity analyses were performed on all first cultures with *Enterobacteriaceae* in 715 individuals with a median age of 71 years (IQR 63–78 years), of whom 74.8% were women (**Tables S3 and S4**). The analysis showed similar results with the analysis in *E. coli*, although the association between a higher intake of beef and trimethoprim resistance was now significant in this analysis. Furthermore, in addition to the association with cefotaxime resistance, a higher intake of chicken was now also associated with resistance to first-generation cephalosporins. Moreover, an inverse association was shown between a higher intake of vegetables and trimethoprim resistance and sulfamethoxazole-trimethoprim resistance (**Table S5**)

Discussion

This epidemiological study in a middle-aged and elderly community-dwelling population showed

Table 1: General characteristics of the study population (n=612) for each antimicrobial drug analysis

	AMX n = 611	AMC n = 612	TMP n = 612	TMP-SFX n = 611	CFZ/CLO n=312	CTX n=462	NIT n=612	NFX n=612
Female sex, n (%)	480(78.6)	481 (78.6)	481 (78.6)	480 (78.6)	253 (81.1)	369 (79.9)	481 (78.6)	481 (78.6)
Age (at culture), median (range)	70 (63-78)	70 (63-78)	70 (63-78)	70 (63-78)	71 (62-78)	73 (65-80)	70 (63-78)	70 (63-78)
History of AB use, median (range)	2 (0 - 4)	2 (0-4)	1 (0-3)	1 (0-3)	0 (0-0)	0 (0-0)	1 (0-2)	0 (0-1)
Diabetes, n (%)	69 (11.3)	69 (11.3)	69 (11.3)	69 (11.3)	44 (14.1)	65 (14.1)	69 (11.3)	69 (11.3)
BMI, median (range)	27.1 (25-30)	27.1 (25-30)	27.1 (25-30)	27.1 (25-30)	27.2 (25-30)	27.2 (24-30)	27.1 (25-30)	27.1 (25-30)
Missing**, n (%)**	23 (3.8)	24 (3.9)	24 (3.9)	24 (3.9)	11 (3.5)	18 (3.9)	24 (3.9)	24 (3.9)
GFR, median (range)	82.9 (72-93)	82.9 (72-93)	82.9 (72-93)	83.0 (72-93)	82.2 (72-91)	83.1 (72-92)	82.9 (72-93)	82.9 (72-93)
Missing, n (%)	50 (8.2)	51 (8.3)	51 (8.3)	51 (8.3)	28 (9.0)	40 (8.7)	51 (8.3)	51 (8.3)
SES, n (%)								
Primary	64 (10.5)	64 (10.5)	64 (10.5)	64 (10.5)	31 (9.9)	51 (11.0)	64 (10.5)	64 (10.5)
Lower	290 (47.5)	291 (47.5)	291 (47.5)	290 (47.5)	157 (50.3)	218 (47.2)	291 (47.5)	291 (47.5)
Intermediate	154 (25.2)	154 (25.2)	154 (25.2)	154 (25.2)	69 (22.1)	118 (25.5)	154 (25.2)	154 (25.2)
Higher	96 (15.7)	96 (15.7)	96 (15.7)	96 (15.7)	49 (15.7)	68 (14.7)	96 (15.7)	96 (15.7)
Missing	7 (1.1)	7 (1.1)	7 (1.1)	7 (1.1)	6 (1.9)	7 (1.5)	7 (1.1)	7 (1.1)
Resistance, n (%)	246 (40.3)	79 (12.9)	167 (27.3)	149 (24.4)	74 (23.7)	16 (3.5)	29 (4.7)	44 (7.2)

Data are median (range) for continuous or the number (%) for categorical variables. The median number of antimicrobial drug prescriptions prior to culture are given for each antimicrobial drug group (ATC code J01CA+ J01CR for amoxicillin and amoxicillin-clavulanic acid, J01EA and J01EE for trimethoprim and sulfamethoxazole-trimethoprim, J01DB for first-generation cephalosporins, J01DD for cefotaxime, J01XE for nitrofurantoin and J01MA for norfloxacin). BMI is body mass index in kg/m^2. GFR is glomerular filtration rate, according to the CKD EPI equation in mL/min per 1.73 m^2. SES is socioeconomic status, (primary = primary education; lower education = lower/intermediate general education or lower vocational education; intermediate = intermediate vocational education or higher general education; higer = higher vocational education or university. Data are given for all studied antimicrobial drugs (AMX = amoxicillin, AMC = amoxicillin-clavulanic acid, TMP = trimethoprim, TMP-SFX = trimethoprim-sulfamethoxazole, CFZ/CLO = first- generation cephalosporins cefazolin and cefalotin, CTX = cefotaxime, NIT = nitrofurantoin, NFX = norfloxacin). The E.coli isolates were mostly tested for their sensitivity to several antimicrobial drugs, meaning that these cultures could be used in several association models.

several associations between diet and the selection of resistant *E. coli* isolates in UTIs. A high pork intake was associated with norfloxacin resistance and a high chicken intake with cefotaxime resistance. On the other hand, inverse associations were also found—a high intake of cheese was, for example, inversely associated with resistance to amoxicillin and amoxicillin-clavulanic acid.

The Rotterdam Study gave us the unique possibility to investigate the associations between diet and antimicrobial resistance in clinical infections by having available urinary cultures and detailed information on diet in the same real-life population. Unfortunately, we could not determine underlying molecular and genetic associations of the resistance genes found in urinary cultures of participants and those found in food animals. Another limitation is the multiple testing in this study, despite restriction of our analyses to eight association models. Although dietary data were collected using self-report, we adjusted the dietary variables for energy intake to account for potential systematic error.[14] Furthermore, the FFQ has been validated in a comparable population and showed adequate ranking of individuals according to their dietary intake.[12,13] Although, we could only use data for one FFQ for this study, which was not taken at the time of urinary culture, it has been shown that most individual food items showed comparable ranking of the participants in the population over time.[18] Also, we adjusted for the time-lag between dietary assessment and culturing. Furthermore, we did not have any information on international travel, which is known to be a risk factor for carriage of antimicrobial resistance, or on the presence of indwelling catheters or stays in a nursing home, but numbers are expected to be very low in this community-dwelling population. However, if none of these factors is associated with diet, they will not have confounded our results. Moreover, the fact that the study population consists of middle-aged and elderly individuals from a specific area of Rotterdam may have affected the generalizability of the results.

The association between a high intake of chicken and cefotaxime resistance in *E. coli* isolates causing UTIs suggests a possible transmission of *E. coli* or of cefotaxime resistance genes via food. Although alarming, this finding was not unexpected, as a Dutch study from 2010 showed that 94% of tested chicken meat samples from retail contained at least one *E. coli* isolate with an extended-spectrum β-lactamase phenotype.[19]

We also showed an association between a high intake of pork and norfloxacin resistance. This confirms the results of our previous study, which showed an association between a high intake of pork and chicken, with ciprofloxacin resistance and which partly used the same cultures.[5] The additional analyses in *Enterobacteriaceae* also showed an association between a high intake of beef and trimethoprim resistance, which was borderline non-significant in *E. coli*, possibly because of a lack of power. Both these results need further investigation as the reported resistance to fluoroquinolones of *E. coli* isolates in pork and to trimethoprim in dairy cows are low, although these numbers were higher in veal calves.[20]

Table 2: Food groups associated with antimicrobial resistance in *E.coli*

	Univariate analysis		Multivariate analysis			
Amoxicillin	**OR**	**p-value**	**OR quartile 2 vs 1**	**OR quartile 3 vs 1**	**OR quartile 4 vs 1**	**OR trend**
Fish	1.15	0.05	-	-	-	-
Cheese	0.86	0.05	0.57 (0.35 – 0.92)*	0.49 (0.30 – 0.79)*	0.57 (0.35 – 0.94)*	0.84 (0.72 – 0.99)*
Potatoes	0.88	0.09	1.07 (0.67 – 1.72)	1.30 (0.81 – 2.07)	0.53 (0.32 – 0.87)*	0.86 (0.74 – 1.01)
Amoxicillin-clavulanic acid						
Shellfish	0.86	0.18	-	-	-	-
Cheese	0.65	<0.005	0.59 (0.32 – 1.11)	0.43 (0.22 – 0.86)*	0.31 (0.14 – 0.68)*	0.67 (0.53 – 0.86)*
Trimethoprim						
Beef	1.13	0.15	1.11 (0.65– 1.91)	1.69 (1.00 – 2.86)*	1.41 (0.83 – 2.43)	1.16 (0.98 – 1.37)
Fruit	1.12	0.18	-	-	-	-
Sulfamethoxazole-trimethoprim						
Beef	1.13	0.15	-	-	-	-
Shellfish	1.14	0.13	-	-	-	-
1st-gen cephalosporins						
Eggs	0.83	0.12	-	-	-	-
Cefotaxime						
Chicken	2.09	0.04	not possible	not possible	NA	2.18 (1.05 – 4.51)*
Offal	0.60	0.13	0.78 (0.24 – 2.55)	0.23 (0.04 – 1.23)	NA	0.52 (0.25 – 1.06)
Cheese	1.66	0.13	0.85 (0.18 – 4.10)	3.25 (0.83 – 12.73)	NA	1.95 (0.95 – 4.00)
Fruit	1.85	0.07	-	-	-	-

Nitrofurantoin						
Offal	1.28	0.15	-	-	-	-
Fish	1.32	0.11	-	-	-	-
Shellfish	1.41	0.05	0.16 (0.02 – 1.35)	1.87 (0.65 – 5.40)	1.70 (0.58 – 4.96)	1.40 (0.97 – 2.02)
Norfloxacin						
Pork	1.27	0.10	0.80 (0.30 – 2.17)	1.17 (0.42 – 3.22)	2.59 (1.01 – 6.61)*	1.42 (1.04 – 1.95)*
Fruit	1.22	0.16	-	-	-	-
Vegetables	0.70	0.01	1.30 (0.58 – 2.94)	0.70 (0.28 – 1.72)	0.34 (0.10 – 1.14)	0.73 (0.53 – 1.01)
Eggs	1.22	0.16	-	-	-	-

Odds ratios (95% CI) and p-values of several food groups per antimicrobial drug (amoxicillin, amoxicillin-clavulanic acid, trimethoprim, sulfamethoxacole-trimethoprim, first-generation cephalosporins, cefotaxime, nitrofurantoin and norfloxacin) per quartile increase. Beacuse of the low numbers of cases for cefotaxime, categorisation was performed in tertiles. The first column shows all ORs of food groups with p<0.2 after univariate analysis and their corresponding p-values. All p-values of the univariate analyses can be found in Table S1. The second column shows the food groups that are included in the multivariate analysis after backward selection with p <0.1, keeping the confounders (sex, age, number of prescriptions (for the specific antibacterial drug group), GFR, diabetes, BMI, SES, follow-up time, time between completing FFQ and urinary culture, and DHD index) in the model independent of their p-value. The analyses for amoxicillin and amoxicillin-clavulanic acid were adjusted for all prescriptions of the ATC-code J01CA and J01CR groups; the analyses for trimethoprim and sulfamethoxazole-trimethoprim were adjusted for all prescriptions of J01EA and J01EE, the analysis for first-generation cephalosporins was adjusted for all prescriptions of J01DB, the analysis for cefotaxime was adjusted for all prescriptions of J01DD, the analysis for nitrofurantoin was adjusted for all prescriptions of J01XE and the analysis for norfloxacin was adjusted for all prescriptions of J01MA.-For all models the p-value of the Hosmer-Lemeshow test of the final model gave p > 0.05, indicating a good fit. Because of the low number of cases, the analysis of cefotaxime was performed in tertiles. Furthermore, not ORs for the quartiles could be given in this analysis, since there were no cases in the lowest tertile.*p-value for OR < 0.05. NA is not applicable.

The possibility that eggs and dairy products play a direct role in the transmission of antimicrobial resistance from animals to humans is not very likely according to the results of this study. Especially dairy products, which are pasteurized before consumption. For crops, it is hypothesized that contaminated water or manure can possibly transfer resistant pathogens, when crop cleaning is performed improperly.[21] However, we could not find any evidence that this results in antimicrobial resistance in UTIs either. In contrast, a higher intake of cheese was associated with less amoxicillin and amoxicillin-clavulanic acid resistance and a higher intake of vegetables was associated with less trimethoprim and sulfamethoxazole-trimethoprim resistance in the sensitivity analyses for *Enterobacteriaceae*. As expected, amoxicillin-clavulanic acid-resistant *E.coli* were also resistant to amoxicillin, which was also true for sulfamethoxazole-trimethoprim-resistant and trimethoprim-resistant *E.coli*, and so these results were comparable. It is not likely that these associations were caused by the fact that individuals compensate a low intake of meat with a high intake of other foods such as cheese and vegetables, as additional adjustment for different meat types did not substantially change the magnitude of these associations.

A hypothesis of the 'protective' effects of these food groups could be that they affect the human gut microbiota. The 'resistome' and dysbiosis of the gut microbiota were hypothesized to contribute in horizontal gene transfer of resistance genes in the gut.[22,23] Moreover, in obese Chinese children, a dietary intervention was shown to diminish the gut resistome.[24] Also, a potential beneficial effect of diets rich in vegetables and cheese on the gut microbiota has been described.[25,26] Interestingly, a small study in healthy volunteers showed lower levels of amoxicillin resistance in Enterococci, but not in *E. coli*, when consuming cheese during treatment with amoxicillin-clavulanic acid.[27] Furthermore, potatoes are the main sources of polysaccharides and fibres,[28] which have been proposed to be beneficial for the gut microbiota.[29-31]

In conclusion, we showed an association between a high intake of chicken and cefotaxime resistance in UTIs caused by *E. coli*, suggesting a possible transfer of antimicrobial resistance from food animals to humans. Surprisingly, we also showed an association between a higher intake of cheese and lower amoxicillin/amoxicillin-clavulanic acid resistance in UTIs, which may be explained by the beneficial effects on the gut microbiota. Further studies on food, microbiota and antimicrobial resistance are necessary to confirm this possible association and unravel underlying mechanisms.

Appendix S1: Method of using dietary data.

Habitual food intake of the participants of The Rotterdam study was assessed with an extensive semi-quantitative FFQ, which consisted of 389 food items. These food items (in grams per day) were divided into the different food groups that we studied (i.e. beef, chicken, pork, offal, fish, shellfish and crustaceans, animal milk products, eggs, cheese, yoghurt and cottage cheese, fruits, vegetables and potatoes). Total energy intake was calculated using the Dutch Food Composition Table of 2006 and 2011. Food group intake was adjusted for total energy intake by using the residual model in order to: 1) remove extraneous variation; 2) remove potential confounding by total energy intake; and 3) account for potential measurement error associated with energy intake (i.e. measurement error increases when energy intake increases). In brief, when using the residual method, the intake of a specific food group by the participants was regressed on the total energy intake, according to the following formula: Food group (Y) = $\beta 0 + \beta 1$*total energy intake. The residuals of this regression were obtained. Because as a definition all residuals together add up to 0, we obtain a ranking of all individuals for each food group. The median intake of the total group was added to these residuals with as a result the intake of the food group fixed to the median energy intake of the population. Finally, in order to eliminate a large effect by outliers, we divided (for all different food groups) all participants in quartiles and studied the effect of the higher quartiles in comparison with the lowest quartile.

Table S1A: ORs (95%CI) and p-values for univariate analysis of *E.coli*

	Amoxicillin	Amoxicillin-clavulanic acid	Trimethoprim	Sulfamethoxazole -trimethoprim	1st generation cephalosporins	Cefotaxime	Nitrofurantoin	Norfloxacin
Beef	0.97 (0.84 – 1.13) p = 0.72	0.93 (0.75 – 1.15) p = 0.48	1.13 (0.96 – 1.33) p = 0.13	1.13 (0.96 – 1.34) p = 0.15	0.92 (0.73 – 1.16) p = 0.48	0.82 (0.44 – 1.52) p = 0.53	0.99 (0.71 – 1.38) p = 0.93	1.06 (0.81 – 1.40) p = 0.68
Chicken	0.99 (0.86 – 1.14) p = 0.87	0.99 (0.81 – 1.23) p = 0.96	1.00 (0.86 – 1.18) p = 0.97	1.00 (0.85 – 1.18) p = 0.99	1.15 (0.91 – 1.46) p = 0.24	2.09 (1.05 – 4.15) p = 0.04	0.90 (0.65 – 1.26) p = 0.55	0.96 (0.73 – 1.27) p = 0.78
Pork	0.97 (0.84 – 1.12) p = 0.65	0.90 (0.72 – 1.11) p = 0.31	1.02 (0.87 – 1.20) p = 0.78	1.05 (0.89 – 1.23) p = 0.60	0.97 (0.77 – 1.23) p = 0.81	0.91 (0.49 – 1.67) p = 0.76	0.96 (0.69 – 1.34) p = 0.80	1.27 (0.96 – 1.68) p = 0.10
Offal	1.00 (0.86 – 1.16) p = 0.99	0.98 (0.80 – 1.21) p = 0.87	1.02 (0.87 – 1.19) p = 0.84	1.09 (0.93 – 1.29) p = 0.29	1.06 (0.84 – 1.34) p = 0.63	0.60 (0.32 – 1.15) p = 0.13	1.28 (0.91 – 1.81) p = 0.15	1.15 (0.87 – 1.51) p = 0.33
Fish	1.15 (1.00 – 1.33) p = 0.05	1.14 (0.93 – 1.42) p = 0.22	1.09 (0.93 – 1.27) p = 0.31	1.10 (0.93 – 1.30) p = 0.26	0.97 (0.77 – 1.23) p = 0.81	1.10 (0.60 – 2.03) p = 0.76	1.32 (0.94 – 1.87) p = 0.11	1.08 (0.82 – 1.42) p = 0.58
Shellfish and crustaceans	1.02 (0.88 – 1.18) p = 0.79	0.86 (0.70 – 1.07) p = 0.18	1.06 (0.90 – 1.24) p = 0.49	1.14 (0.96 – 1.25) p = 0.13	0.92 (0.73 – 1.16) p = 0.48	0.67 (0.36 – 1.27) p = 0.22	1.41 (0.99 – 2.00) p = 0.05	0.96 (0.73 – 1.27) p = 0.78
Animal milk products	0.98 (0.85 – 1.14) p = 0.81	0.97 (0.79 – 1.20) p = 0.79	1.03 (0.88 – 1.21) p = 0.72	1.01 (0.85 – 1.19) p = 0.93	0.89 (0.71 – 1.13) p = 0.34	0.67 (0.36 – 1.27) p = 0.22	0.99 (0.71 – 1.38) p = 0.93	0.94 (0.72 – 1.24) p = 0.68
Eggs	1.07 (0.93 – 1.24) p = 0.37	1.10 (0.89 – 1.37) p = 0.36	1.04 (0.88 – 1.22) p = 0.66	1.01 (0.86 – 1.20) p = 0.87	0.83 (0.66 – 1.05) p = 0.12	0.91 (0.49 – 1.67) p = 0.76	1.08 (0.77 – 1.50) p = 0.67	1.22 (0.92 – 1.61) p = 0.16
Cheese	0.86 (0.75 – 1.00) p = 0.05	0.65 (0.52 – 0.81) p < 0.005	0.95 (0.81 – 1.12) p = 0.54	0.98 (0.83 – 1.16) p = 0.79	1.03 (0.82 – 1.30) p = 0.81	1.66 (0.86 – 3.16) p = 0.13	1.25 (0.89 – 1.75) p = 0.21	1.06 (081 – 1.40) p = 0.68
Yoghurt and cottage cheese	0.97 (0.84 – 1.12) p = 0.65	1.01 (0.81 – 1.24) p = 0.96	1.01 (0.86 – 1.18) p = 0.90	1.00 (0.85 – 1.18) p = 0.99	0.97 (0.77 – 1.23) p = 0.81	1.34 (0.72 – 2.51) p = 0.35	0.83 (0.59 – 1.16) p = 0.83	1.17 (0.89 – 1.54) p = 0.26
Fruits	1.03 (0.90 – 1.20) p = 0.65	1.14 (0.93 – 1.42) p = 0.22	1.12 (0.95 – 1.31) p = 0.18	1.03 (0.87 – 1.22) p = 0.74	1.11 (0.87 – 1.40) p = 0.41	1.85 (0.95 – 3.60) p = 0.07	1.01 (0.73 – 1.42) p = 0.93	1.22 (0.92 – 1.61) p = 0.17
Vegetables	0.99 (0.86 – 1.15) p = 0.92	1.02 (0.82 – 1.26) p = 0.87	0.92 (0.78 – 1.07) p = 0.27	0.92 (0.78 – 1.09) p = 0.36	0.91 (0.72 – 1.14) p = 0.41	1.10 (0.60 – 2.03) p = 0.76	0.90 (0.65 – 1.26) p = 0.55	0.70 (0.52 – 0.93) p = 0.01
Potatoes	0.88 (0.76 – 1.02) p = 0.09	1.03 (0.83 – 1.27) p = 0.79	1.07 (0.91 – 1.26) p = 0.39	1.04 (0.88 – 1.23) p = 0.63	1.09 (0.86 – 1.38) p = 0.48	1.00 (0.54 – 1.84) p = 1.00	1.11 (0.79 – 1.55) p = 0.55	0.94 (0.72 – 1.24) p = 0.68

Table S2: Spearman's correlation coefficients for food groups

Food group	Beef	Chicken	Pork	Offal	Fish	Shellfish and crustaceans	Animal milk products	Eggs	Cheese	Yoghurt and cottage cheese	Fruits	Vegetables	Potatoes
Beef	-	0.25	0.65	0.15	0.16	0.11	0.00	0.13	0.15	0.02	0.01	0.20	0.15
Chicken		-	0.25	0.03	0.21	0.02	-0.05	0.14	0.10	0.08	0.13	0.27	-0.01
Pork			-	0.18	0.07	0.08	0.04	0.18	0.21	-0.03	0.02	0.11	0.17
Offal				-	0.05	0.07	0.09	0.16	0.10	0.03	-0.06	0.00	0.06
Fish					-	0.24	0.03	0.16	0.06	0.07	0.14	0.25	0.01
Shellfish and crustaceans						-	0.01	0.03	0.02	-0.05	-0.02	0.13	0.06
Animal milk products							-	0.13	0.07	0.00	0.11	-0.08	0.10
Eggs								-	0.11	0.11	0.10	0.12	0.12
Cheese									-	0.07	0.05	0.17	0.04
Yoghurt and cottage cheese										-	0.22	0.20	-0.01
Fruits											-	0.21	-0.01
Vegetables												-	0.13
Potatoes													-

Spearman's correlation coefficients for the different food groups. The coefficients were determined on all individuals with at least one culture with an *E.coli* isolate (n=612). No correlation > 0.7 was observed.

Table S3: General characteristics of the study population (n=715) per antimicrobial drug (*Enterobacteriaceae*)

	AMX n = 653	**AMC n = 707**	**TMP n = 715**	**TMP-SFX n = 714**	**CFZ/CLO n=355**	**CTX n=544**	**NIT n=679**	**NFX n=715**
Female sex, n (%)	498 (76.3)	533 (75.4)	535 (74.8)	534 (74.8)	274 (77.2)	412 (75.7)	519 (76.4)	535 (74.8)
Age (at culture), median (range)	70 (63-78)	71 (63–78)	71 (63-78)	71 (63-78)	71 (63-79)	73 (65-80)	71 (63-78)	71 (63-78)
Prescriptions, median (range)	2 (0-4)	2 (0–4)	1 (0-2)	1 (0-2)	0 (0-0)	0 (0-0)	1 (0-2)	0 (0-1)
Diabetes, n (%)	79 (12.1)	88 (12.4)	89 (12.4)	89 (12.5)	52 (14.6)	85 (15.6)	78 (11.5)	89 (12.4)
BMI, median (range)	27.2 (25-30)	27.2 (24-30)	27.2 (24-30)	27.2 (24-30)	27.3 (25-30)	27.4 (25-30)	27.2 (24-30)	27.2 (24-30)
Missing, n (%)	25 (3.8)	28 (4.0)	29 (4.1)	29 (4.1)	14 (3.9)	25 (4.6)	26 (3.8)	29 (4.1)
GFR, median (range)	82.8 (72-93)	82.9 (72-92)	82.9 (72-92)	82.9 (72-92)	82.3 (72-91)	82.6 (72-92)	83.1 (72-93)	82.9 (72-92)
Missing, n (%)	55 (8.4)	59 (8.3)	63 (8.8)	63 (8.8)	32 (9.1)	50 (9.2)	58 (8.5)	63 (8.8)
SES, n (%)								
Primary	68 (10.4)	74 (10.5)	75 (10.5)	75 (10.5)	36 (10.1)	63 (11.6)	71 (10.5)	75 (10.5)
Lower	307 (47.0)	334 (47.2)	338 (47.3)	337 (47.2)	179 (50.4)	253 (46.5)	324 (47.7)	338 (47.3)
Intermediate	165 (25.3)	178 (25.2)	179 (25.0)	179 (25.1)	78 (22.0)	139 (25.6)	170 (25.0)	179 (25.0)
Higher	106 (16.2)	114 (16.1)	116 (16.2)	116 (16.2)	56 (15.8)	82 (15.1)	107 (15.8)	116 (16.2)
Missing	7 (1.1)	7 (1.0)	7 (1.0)	7 (1.0)	6 (1.7)	7 (1.3)	7 (1.0)	7 (1.0)
Overall resistance, n (%)	255 (39.1)	85 (12.0)	186 (26.0)	166 (23.2)	74 (20.8)	18 (3.3)	93 (13.7)	44 (6.2)
***E.coli*,** n (%)	589 (90.2)	567 (80.2)	559 (78.2)	558 (78.2)	288 (81.1)	418 (76.8)	580 (85.4)	558 (78.0)
Resistance, n (%)	236 (40.1)	72 (12.7)	156 (21.8)	139 (19.5)	70 (19.7)	16 (2.9)	28 (4.1)	41 (7.3)
***Klebsiella spp.*,** n (%)	Excl	67 (9.5)	66 (9.2)	66 (9.2)	31 (8.7)	55 (10.1)	71 (10.4)	66 (9.2)
Resistance, n (%)	NA	3 (4.5)	9 (13.6)	7 (10.6)	2 (6.5)	1 (1.8)	52 (73.2)	0 (0.0)
***P. mirabilis*,** n (%)	64 (9.8)	61 (8.6)	59 (8.3)	59 (8.3)	28 (7.9)	47 (8.6)	Excl	59 (8.3)
Resistance, n (%)	19 (29.7)	9 (1.5)	19 (32.2)	18 (30.5)	2 (7.1)	0 (0.0)	NA	2 (3.4)
Other *proteus spp.*, n (%)	Excl	-	-	-	Excl	-	Excl	-
Resistance, n (%)	NA	-	-	-	NA	-	NA	-
***Citrobacter freundii*,** n (%)	Excl	Excl	1 (0.1)	1 (0.1)	Excl	2 (0.4)	2 (0.3)	1 (0.1)
Resistance, n (%)	NA	NA	1 (100)	1 (100)	NA	0 (0.0)	1 (50.0)	1 (100)
Other *citrobacter spp.*	Excl	11 (1.6)	12 (1.7)	12 (1.7)	7 (2.0)	9 (1.7)	12 (1.8)	12 (1.7)
Resistance, n (%)	NA	1 (9.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)

Enterobacter spp., n (%)	Excl	Excl	13 (1.8)	13 (1.8)	Excl	6 (1.1)	13 (1.9)	13 (1.8)
Resistance, n (%)	NA	NA	1 (7.8)	1 (7.8)	NA	1 (16.7)	11 (84.7)	0 (0.0)
Raoultella spp., n (%)	Excl	1 (0.1)	1 (0.1)	1 (0.1)	1 (0.3)	1 (0.2)	1 (0.1)	1 (0.1)
Resistance, n (%)	NA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Morganella spp., n (%)	Excl	Excl	3 (0.4)	3 (0.4)	Excl	4 (0.7)	Excl	3 (0.4)
Resistance, n (%)	NA	NA	0 (0.0)	0 (0.0)	NA	0 (0.0)	NA	0 (0.0)
Serratia spp., n (%)	Excl	Excl	1 (0.1)	1 (0.1)	Excl	2 (0.4)	Excl	1 (0.1)
Resistance, n (%)	NA	NA	0 (0.0)	0 (0.0)	NA	0 (0.0)	NA	0 (0.0)

Data are median (range) for continuous or the number (%) for categorical variables. BMI is body mass index in kg/m^2. GFR is glomerular filtration rate, according to the CKD-EPI equation in mL/min per 1.73 m^2. SES is socioeconomic status, (primary = primary education; lower education = lower/intermediate general education or lower vocational education; intermediate = intermediate vocational education or higher general education; higer = higher vocational education or university).

Data are given for all studied antimicrobial drugs (AMX = amoxicillin, AMC = amoxicillin-clavulanic acid, TMP = trimethoprim, TMP-SFX = trimethoprim-sulfamethoxazole, CFZ/CLO = first generation cephalosporins cefazolin and cefalotin, CTX = cefotaxime, NIT = nitrofurantoin, NFX = norfloxacin). Most pathogens were tested for their sensitivity to several antimicrobial drugs, meaning that these cultures could be used in several association models. Excl = excluded because of intrinsic resistance. "-" = not present. NA is not applicable.

Table S4A: ORs (95%CI) and p-values for univariate analysis of *Enterobacteriaceae*

	Amoxicillin	Amoxicillin-clavulanic acid	Trimethoprim	Sulfamethoxazole-trimethoprim	1st generation cephalosporins	Cefotaxime	Nitrofurantoin	Norfloxacin
Beef	1.01 (0.88 – 1.17) p = 0.85	0.96 (0.79 – 1.18) p = 0.71	1.17 (1.01 – 1.36) p = 0.04	1.15 (0.98 – 1.34) p = 0.08	0.90 (0.72 – 1.13) p = 0.37	0.92 (0.52 – 1.63) p = 0.77	0.97 0.80 – 1.18) p = 0.79	1.06 (0.81 – 1.39) p = 0.68
Chicken	0.98 (0.85 – 1.13) p = 0.79	1.01 (0.83 – 1.24) p = 0.89	1.02 (0.88 – 1.19) p = 0.75	1.06 (0.91 – 1.24) p = 0.48	1.22 (0.96 – 1.53) p = 0.10	2.12 (1.10 – 4.06) p = 0.02	0.86 (0.70 – 1.04) p = 0.12	1.02 (0.78 – 1.34) p = 0.78
Pork	0.97 (0.84 – 1.11) p = 0.63	0.95 (0.78 – 1.17) p = 0.63	1.07 (0.92 – 1.24) p = 0.41	1.10 (0.94 – 1.29) p = 0.23	0.95 (0.75 – 1.19) p = 0.63	0.92 (0.52 – 1.63) p = 0.77	0.91 (0.75 – 1.11) p = 0.34	1.29 (0.98 – 1.71) p = 0.07
Offal	0.97 (0.85 – 1.12) p = 0.99	1.00 (0.81 – 1.22) p = 0.96	1.01 (0.87 – 1.17) p = 0.93	1.07 (0.91 – 1.24) p = 0.43	1.15 (0.92 – 1.45) p = 0.23	0.84 (0.47 – 1.50) p = 0.56	0.96 (0.79 – 1.17) p = 0.67	1.12 (0.86 – 1.48) p = 0.40
Fish	1.14 (0.99 – 1.32) p = 0.07	1.12 (0.91 – 1.37) p = 0.28	1.14 (0.98 – 1.32) p = 0.09	1.16 (0.99 – 1.35) p = 0.07	1.06 (0.84 – 1.33) p = 0.65	1.00 (0.58 – 1.78) p = 1.00	1.09 (0.90 – 1.33) p = 0.39	1.10 (0.84 – 1.45) p = 0.48
Shellfish and crustaceans	1.04 (0.91 – 1.20) p = 0.55	0.95 (0.77 – 1.16) p = 0.58	1.03 (0.89 – 1.20) p = 0.67	1.10 (0.94 – 1.29) p = 0.23	0.96 (0.76 – 1.21) p = 0.72	0.70 (0.39 – 1.27) p = 0.24	1.04 (0.85 – 1.26) p = 0.72	0.94 (0.72 – 1.24) p = 0.67
Animal milk products	1.02 (0.88 – 1.17) p = 0.81	0.96 (0.79 – 1.18) p = 0.70	1.03 (0.89 – 1.20) p = 0.71	1.01 (0.86 – 1.18) p = 0.94	0.94 (0.74 – 1.18) p = 0.57	0.64 (0.35 – 1.17) p = 0.15	1.00 (0.82 – 1.21) p = 0.97	0.91 (0.69 – 1.19) p = 0.48
Eggs	1.00 (0.86 – 1.14) p = 0.87	1.02 (0.83 – 1.24) p = 0.88	0.95 (0.82 – 1.11) p = 0.54	0.92 (0.79 – 1.08) p = 0.30	0.84 (0.66 – 1.06) p = 0.13	1.00 (0.56 – 1.78) p = 1.00	1.02 (0.84 – 1.24) p = 0.67	1.04 (0.79 – 1.37) p = 0.16
Cheese	0.83 (0.72 – 0.96) p = 0.01	0.68 (0.55 – 0.84) p < 0.005	0.89 (0.77 – 1.04) p = 0.13	0.93 (0.80 – 1.09) p = 0.38	1.08 (0.86 – 1.35) p = 0.53	1.42 (0.79 – 2.57) p = 0.13	1.00 (0.82 – 1.21) p = 0.98	1.04 (0.79 – 1.37) p = 0.78
Yoghurt and cottage cheese	1.05 (0.91 – 1.20) p = 0.53	1.18 (0.96 – 1.45) p = 0.11	1.06 (0.91 – 1.23) p = 0.45	1.03 (0.88 – 1.21) p = 0.69	0.97 (0.79 – 1.25) p = 0.27	1.42 (0.79 – 2.57) p = 0.24	0.84 (0.69 – 1.02) p = 0.08	1.24 (0.94 – 1.64) p = 0.26
Fruits	1.04 (0.90 – 1.19) p = 0.63	1.15 (0.94 – 1.42) p = 0.17	1.10 (0.94 – 1.27) p = 0.23	1.05 (0.90 – 1.23) p = 0.27	1.12 (0.89 – 1.41) p = 0.41	1.42 (0.79 – 2.57) p = 0.07	1.11 (0.91 – 1.35) p = 0.29	1.12 (0.85 – 1.48) p = 0.41
Vegetables	0.96 (0.84 – 1.11) p = 0.92	1.08 (0.88 – 1.33) p = 0.44	0.89 (0.77 – 1.03) p = 0.12	0.89 (0.76 – 1.04) p = 0.13	0.91 (0.72 – 1.15) p = 0.42	1.19 (0.67 – 2.12) p = 0.56	0.79 (0.65 – 0.97) p = 0.02	0.68 (0.51 – 0.91) p = 0.01
Potatoes	0.87 (0.76 – 1.01) p = 0.06	0.91 (0.75 – 1.12) p = 0.38	1.05 (0.90 – 1.22) p = 0.54	1.01 (0.87 – 1.18) p = 0.87	1.08 (0.86 – 1.35) p = 0.53	0.92 (0.52 – 1.63) p = 0.77	1.12 (1.00 – 1.49) p = 0.05	0.93 (0.71 – 1.22) p = 0.58

Table S5: Food groups associated with antimicrobial resistance in *Enterobacteriaceae*

	Univariate analysis		Model			
Amoxicillin	**OR**	**p-value**	**OR quartile 2 vs 1**	**OR quartile 3 vs 1**	**OR quartile 4 vs 1**	**OR trend**
Fish	1.14 (0.99 – 1.31)	0.07	-	-	-	-
Cheese	0.83 (0.72 – 0.96)*	0.01	0.53 (0.33 – 0.84)*	0.44 (0.27 – 0.70)*	0.49 (0.30 – 0.79)*	0.80 (0.69 – 0.93)*
Potatoes	0.87 (0.76 – 1.01)	0.06	1.05 (0.66 – 1.67)	1.15 (0.73 – 1.82)	0.51 (0.31 – 0.84)*	0.85 (0.73 – 0.98)*
Amoxicillin-clavulanic acid						
Cheese	0.68 (0.55 – 0.84)*	<0.005	0.55 (0.30 – 1.01)	0.49 (0.26 – 0.91)*	0.31 (0.15 – 0.65)*	0.69 (0.55 – 0.86)*
Yoghurt/cottage cheese	1.18 (0.96 – 1.45)	0.11	-	-	-	-
Fruits	1.15 (0.94 – 1.42)	0.17	-	-	-	-
Trimethoprim						
Beef	1.17 (1.01 – 1.36)*	0.04	1.61 (0.96 – 2.72)	1.91 (1.14 – 3.21)*	1.79 (1.05 – 3.05)*	1.20 (1.02 – 1.42)*
Fish	1.14 (0.98 – 1.32)	0.09	-	-	-	-
Cheese	0.89 (0.77 – 1.04)	0.13	0.70 (0.43 – 1.13)	0.51 (0.31 – 0.85)*	0.72 (0.43 – 1.19)	0.87 (0.74 – 1.03)
Vegetables	0.89 (0.76 – 1.03)	0.12	0.67 (0.41 – 1.10)	0.88 (0.54 – 1.44)	0.56 (0.33 – 0.95)*	0.87 (0.73 – 1.03)
Sulfamethoxazole-trimethoprim						
Beef	1.15 (0.98 – 1.34)	0.08	1.48 (0.86 – 2.55)	1.55 (0.91 – 2.66)	1.60 (0.92 – 2.79)	1.16 (0.98 – 1.37)
Fish	1.16 (0.99 – 1.35)	0.07	1.06 (0.62 – 1.81)	1.21 (0.71 – 2.07)	1.60 (0.94 – 2.71)	1.17 (0.99 – 1.38)
Vegetables	0.89 (0.76 – 1.04)	0.13	0.64 (0.38 – 1.08)	0.81 (0.49 – 1.35)	0.49 (0.28 – 0.87)*	0.84 (0.70 – 1.00)*
First-generation cephalosporins						
Chicken	1.22 (0.96 – 1.53)	0.10	2.04 (0.83 – 5.00)	1.70 (0.67 – 4.30)	2.80 (1.13 – 6.97)*	1.34 (1.01 – 1.77)*
Eggs	0.84 (0.66 – 1.06)	0.13	0.51 (0.22 – 1.17)	0.64 (0.29 – 1.43)	0.39 (0.16 – 0.93)*	0.78 (0.59 – 1.02)
Cefotaxime						
Chicken	2.12 (1.10 – 4.06)*	0.02	8.85 (1.21 – 64.54)*	10.03 (1.52 –66.15)*	NA	2.16 (1.11– 4.23)*
Animal milk products	0.64 (0.35 – 1.17)	0.15	-	-	NA	-

Nitrofurantoin						
Chicken	0.86 (0.70 – 1.04)	0.12	-	-	-	-
Yoghurt/cottage cheese	0.84 (0.69 – 1.02)	0.08	-	-	-	-
Potatoes	1.22 (1.00 – 1.49)	0.05	-	-	-	-
Vegetables	0.79 (0.65 – 0.97)*	0.02	-	-	-	-
Norfloxacin						
Pork	1.29 (0.98 – 1.71)	0.07	1.10 (0.41 – 2.95)	1.03 (0.35 – 2.97)	2.90 (1.11 – 7.59)*	1.43 (1.04 – 1.98)*
Yoghurt/cottage cheese	1.24 (0.94 – 1.64)	0.13	0.63 (0.22 – 1.82)	1.40 (0.55 – 3.58)	2.02 (0.80 – 5.10)	1.32 (0.97 – 1.80)
Vegetables	0.68 (0.51 – 0.91)*	0.01	1.12 (0.49 – 2.56)	0.68 (0.27 – 1.72)	0.36 (0.11 – 1.20)	0.74 (0.53 – 1.02)

Odds ratios (95% CI) and p-values of several food groups per antimicrobial drug (amoxicillin, amoxicillin-clavulanic acid, trimethoprim, sulfamethoxacole-trimethoprim, first-generation cephalosporins, cefotaxime, nitrofurantoin and norfloxacin) per quartile increase. The first column shows all food groups with $p<0.2$ after univariate analysis. The second column shows the food groups that are included in the model after backward selection with $p<0.1$, keeping the confounders (sex, age, number of prescriptions, GFR, diabetes, BMI, SES, follow-up time, time between completing FFQ and urinary culture and DHD index) in the model independent of their p-value. The analyses for amoxicillin and amoxicillin-clavulanic acid were adjusted for all prescriptions of the ATC-code J01CA and J01CR groups; the analyses for trimethoprim and sulfamethoxazole-trimethoprim were adjusted for all prescriptions of J01EA and J01EE, the analysis for first-generation cephalosporins was adjusted for all prescriptions of J01DB, the analysis for cefotaxime was adjusted for all prescriptions of J01DD, the analysis for nitrofurantoin was adjusted for all prescriptions of J01XE and the analysis for norfloxacin was adjusted for all prescriptions of J01MA. For all models the p-value of the Hosmer-Lemeshow test of the final model gave $p > 0.05$, indicating a good fit.*p-value for OR < 0.05. NA is not aplicable.

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Chapter 4

Antimicrobial Drugs, Antimicrobial Resistance, Urinary Tract Infections and the Microbiota

Chapter 4.1

The Effect of Antimicrobial Drug Use on the Composition of the Genitourinary Microbiota in an Elderly Population.

Marlies Mulder, Djawad Radjabzadeh, Robert-Jan Hassing, Jan Heeringa, Andre Uitterlinden, Robert Kraaij, Bruno H Stricker, Annelies Verbon

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Abstract

Objective The urinary tract is inhabited by a diversity of microorganisms, known as the genitourinary microbiota. Here, we investigated the association between the use of antimicrobial drugs and the composition of the genitourinary microbiota.

Methods Clean-catch urinary samples were collected from 27 participants of the Rotterdam Study. Bacterial DNA was extracted and the 16S ribosomal RNA gene variable regions V3 and V4 were analyzed using Illumina sequencing. 23 of the 27 participants were included in the analysis.

Results The population consisted of 10 men and 13 women with a mean age of 75 ± 3 years. The time between the last prescription of an antimicrobial drug and sampling was determined and categorized. The use of antimicrobial drugs prior to urine sampling was associated with statistically significant differences in the beta-diversity of the genitourinary microbiota. No association was found between antimicrobial drug use and the alpha-diversity of the genitourinary microbiota. Operational Taxonomic Units (OTUs) that were lowest in participants who used antimicrobial drug belonged to Lactobacillus and Finegoldia. In contrast, an OTU belonging to the genus Parabacteroides had higher abundances. Also, an OTU belonging to the species *E.coli* was higher in the participants who used antimicrobial drugs.

Conclusions Prior use of antimicrobial drugs is associated with a different composition of the genitourinary microbiota. Our results might indicate a persisting effect of antimicrobial drugs on the composition of the microbiota, but reverse causality cannot be ruled out. Future studies are needed to differentiate between two possibilities. Genitourinary dysbiosis could be the result of antimicrobial drug use or genitourinary dysbiosis could be a risk factor for urinary tract infections resulting in increased use of antimicrobial drugs. This may have important implications for treatment and prevention of (recurrent) UTIs.

Introduction

The term microbiota, which is often interchangeably used with the microbiome, is defined as the microorganisms that live in a particular body compartment.[1] The microbiota of the gut are the most well-known microbiota and have been described in many studies. Currently, we know that several other body compartments, such as the skin, nose and urinary tract, also have a distinct microbiota and it is assumed that these microbiota are associated with overall health.[2]

For long, it was thought that urine was sterile, and the presence of microorganisms in the urinary tract was considered to occur only as part of an infection. In 1979, it was recognized that slow-growing micro-organisms were missed when standard culturing techniques were used.[3] However, it was only with the development of 16S ribosomal RNA sequencing that it was established that most body sites are colonized with bacteria, but the urinary tract was not tested in the Human Microbiome Project.[4] Recently, the microbiota unique to the urinary tract have been reported both in males and females.[5,6] In females, the microbiota seem to be more complex with higher interindividual variability than in males,[7] but no clear relation with urinary tract infections (UTIs) has been demonstrated until now. No evident core microbiota have been found yet, however, this could possibly be present when grouping by age.[7,8]

With the discovery of the urinary microbiota, the interest is growing. Until now, most studies have included small numbers of individuals and have shown considerable variation in the (genito)urinary microbiota within the study population. Nevertheless, several studies have already suggested a dysbiosis of the (genito)urinary microbiota in diseases such as urgency urinary incontinence.[9-12]

Another factor that might influence the genitourinary tract microbiota is the use of antimicrobial drugs. For the gut microbiota, it has already been shown that use of antimicrobial drugs (temporarily) influences the composition of the microbiota.[13-15] This effect has not yet been demonstrated for the genitourinary microbiota, despite the fact that antibiotic drugs are very often prescribed for urinary tract infections and have a good penetration in the urinary tract. Here, we investigated the association between the use of antimicrobial drugs and the composition of the genitourinary microbiota.

Methods

Source population

Twenty-seven participants were randomly selected from the Rotterdam Study, a prospective population-based cohort study of middle-aged and elderly people in the Ommoord area of Rotterdam,[27] were asked to provide a urine sample (November/December 2015). The Rotterdam

Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, on the basis of the Wet Bevolkingsonderzoek ERGO. All participants provided written informed consent.

Microbiome analysis

Samples of first-morning clean-catch midstream urine (~ 50 mL) were collected and centrifuged at 6000 g for 10 min. Supernatants were removed and pellets were resuspended in the remaining urine and stored at − 80 °C. Automated DNA-isolation (Arrow DNA; DiaSorin S.p.A., Saluggia, Italy) was performed using the Arrow DNA kit according to the manufacturer's instructions and included bead-beating in Lysing Matrix B tubes containing 0.1 mm silica beads (MP Biomedicals, LLC, Bio Connect Life Sciences, Huissen, The Netherlands) using the MagNA Lyser instrument (Roche Diagnostics, Almere, The Netherlands) at 7000 rpm for 45 s. Bacterial 16S rRNA variable regions V3 and V4 were amplified and sequenced using the Illumina MiSeq 2 × 300 base pairs protocol.[28] Phylogenetic multi-sample profiling was performed using an in-house developed pipeline based on the QIIME 1.9.0 and USEARCH version 8.1 software packages.[29,30] After rarefaction at 10,000 reads per sample, taxonomy was assigned both at genus and species level using the naïve Bayesian RDP classifier[31] and the SILVA database (v119).[32] Cluster analysis and *MaAsLin* analysis were performed on the dataset at genus level. For heatmap analysis, the Operational Taxonomic Unit (OTU) table was cleaned; singletons and OTUs with minimum count fraction of 0.005%[33] (50 reads) were discarded. In addition, the 4% of genera with the lowest abundance were removed.

Antimicrobial drug use

The date of the last prescription and the total number of prescriptions before sampling of several antimicrobial drug groups was obtained from a collaborative database of all community pharmacies in the Ommoord area. This included: tetracyclines (J01A), beta-lactams (J01C), sulphonamides and trimethoprim (J01E), macrolides (J01FA), fluoroquinolones (J01MA), nitrofuran derivatives (J01XE) and fosfomycin (J01XX01). Although the proportion of renal excretion differs, all of these antimicrobial drugs have a substantial excretion via urine. Macrolides (J01FA) were only analyzed in a sensitivity analysis because they are mainly excreted by the gallbladder. Cephalosporins and aminoglycosides were not prescribed. The time between the last prescription of one of these drugs and urinary sampling was calculated and categorized into no use (0), use > 96 months before sampling (1), use 73–96 months before sampling (2), use 49–72 months before sampling (3), use 25–48 months before sampling (4), use 13–24 months before sampling (5), use 0–12 months before sampling (6).

Analysis and statistical methods

Statistical analyses were performed in R.[34] Shannon alpha-diversities (measure of diversity of species within a sample) and Bray-Curtis beta-diversities (measure of diversity of species composition between samples) were calculated. Differences between users of antimicrobial

drugs in alpha-diversities were tested using a linear regression analysis with the Shannon alpha-diversity as the response variable and the time category of last antimicrobial drug use as the explanatory variable. Differences in beta-diversities were tested using the MiRKAT package, in which it is possible to test the association between a microbiome community and a phenotype with the aid of semi-parametric kernel machine regression.[35] The analyses on the alpha-diversity and beta-diversity were adjusted for age, sex, diabetes (use of anti-diabetic medication) and kidney function (glomerular filtration rate (GFR) according to the CKD-EPI equation).[36] In a sensitivity analysis, an additional confounder was included which gave the number of prescriptions of all antimicrobial drugs since start of the collaborative drug database (1st January 1995). The MaAsLin package was used to determine genera that caused the largest differences between groups.[37] In this analysis, the time category of antimicrobial drug use was linearly included as variable of interest, and age, sex, diabetes and kidney function were included as confounders. A p-value < 0.05 was considered statistically significant.

Results

Sufficient bacterial DNA could be obtained in 24 of the 27 participants (88.9%), whereas 3 participants (two males, one female) were excluded from the analysis because the DNA obtained was not sufficient for the analyses. Additionally, 1 participant was excluded because the composition of her microbiota consisted for 99.9% of *Escherichia coli.* Unfortunately, we do not know whether she had symptoms indicating a UTI, thus we could not exclude a UTI at the time of sampling. Therefore, the study population consisted of 10 (45.5%) males and 13 (56.5%) females with a median age of 75 years (range 71–83 years). Of all participants, 7 (30.4%) had used antimicrobial drugs in the previous year (**Table 1**). The microbiota compositions showed considerable variability between participants. The most abundantly detected phyla were Firmicutes, Bacteroidetes and Proteobacteria, in descending order, whereas the most abundant species was *Escherichia coli*. We did not find any differences in alpha-diversity and beta-diversity between men and women.

Also, no difference could be demonstrated in alpha-diversities for antimicrobial drug use, indicating that the diversity of the microbiota was not different in the participants that had used antimicrobial drug use. However, the beta-diversity after (categorized) antimicrobial drug use was significantly different (p < 0.005), meaning that the use of antimicrobial drugs is clearly associated with a different composition of the microbiota. This difference was still present after adjustment for sex, age, diabetes and kidney function (p < 0.005) (**Figure. 1 and Table 2**).

Since other antimicrobial drug prescriptions prior to the last prescription before sampling could also have influenced the genitourinary microbiota, a confounder that represented the number

Table 1: Basic characteristics of the study population

Characteristic	Value
Age, median (IQR)	74.7 (73.1 – 77.0)
Sex (female), n (%)	13 (56.5)
Diabetes, n (%)	4 (17.4)
Kidney function (GFR), median (IQR)	84.4 (74.5 – 94.6)
Antimicrobial drug use, n (%)	
no use	2 (8.7)
>96 months	4 (17.4)
73-96 months	1 (4.3)
49-72 months	1 (4.3)
25-48 months	3 (13.0)
13-24 months	5 (21.7)
0-12 months	7 (30.4)

Table 1 shows the basic characteristics of the study population. Diabetes was assessed as the use of antidiabetic medication. The kidney function indicated the glomerular filtration rate, which was calculated with the *CKD-EPI equation.* The use of any antimicrobial drug (except for J01F) before sampling was categorized in 0-12 months (6), 13-24 months (5), 25-48 months (4), 49-72 months (3), 73-96 months (2) and >96 months (1) before sampling or no use (0).

of other antimicrobial drug prescriptions since approximately 1995 was added in a sensitivity analysis. This did not influence the results ($p < 0.005$). In another sensitivity analysis where macrolides were added to antimicrobial drug use, the community structure was also different after antimicrobial drug use ($p = 0.03$) and borderline significant after adjustment for confounders ($p = 0.05$).

Several OTUs were shown to be significantly lower or higher in participants who used antimicrobial drugs than in those who did not. The OTUs that were most reduced in participants who had used antimicrobial drugs belonged to the genera *Lactobacillus* and a *Finegoldia,* followed by an uncultured member of FamilyXI, 2 OTUs belonging to the genus *Helcococcus*, an OTU belonging to the genus *Gallicola*, 2 OTUs belonging to the genus *Streptococcus and* 2 OTUs belonging to the genus *Porphyromonas*. Of the OTUs that were higher after antimicrobial drug use, one of the strongest belonged to the genus *Escherichia*. Other OTUs that were higher included members of the genera *Parabacteroides*, *Bacteroides* and *Faecalibacterium* as well as uncultured members of the families Ruminococcaceae and Defluviitaleaceae (**Table 2**).

Discussion

In this study of elderly asymptomatic persons, we showed that previous use of antimicrobial drugs is associated with differences in the composition of the genitourinary microbiota. OTUs that were lowest in participants who used antimicrobial drugs belonged to the genera

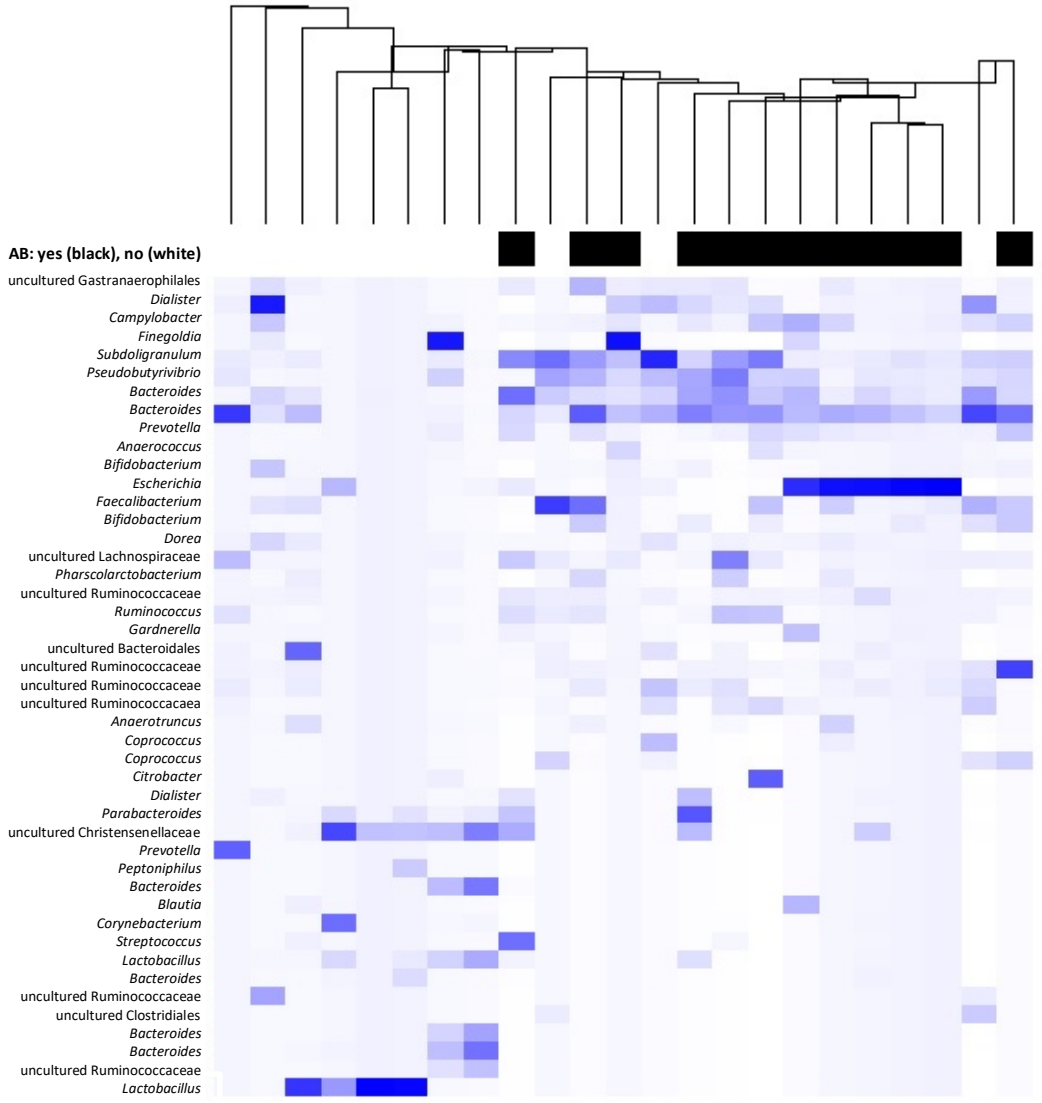

Figure 1. Heatmap of urinary microbiota. Heatmap of bacterial profiles of individuals who used antimicrobial drugs in 48 months before (**black**) and individuals who used no antimicrobial drugs or longer than 48 months before sampling (**white**). These analyses were performed on a cleaned dataset, showing the most abundant OTUs. Bray-Curtis dissimilarities were used to determine differences and median linkage was used for hierarchical clustering of samples.

Lactobacillus and a *Finegoldia.* In contrast, an OTU belonging to the species *E.coli* was higher in participants who used antimicrobial drugs.

We here showed a difference in the genitourinary microbiota after the use of antimicrobial drugs. We only considered the last prescription before urinary sampling, which most likely has

Table 2 Genera that differ after antimicrobial drug use.

Lower after antimicrobial drug use		Higher after antimicrobial drug use	
Genus	**Estimate**	**Genus**	**Estimate**
Lactobacillus	-0.054	*Parabacteroides*	0.046
Finegoldia	-0.054	uncultured Ruminococcaceae	0.041
uncultured FamilyXI	-0.053	*Bacteroides*	0.039
Helcococcus	-0.053	uncultured Defluviitaleaceae	0.037
Gallicola	-0.052	*Escherichia*	0.036
Helcococcus	-0.051	*Faecalibacterium*	0.036
Streptococcus	-0.051	uncultured Ruminococcaceae	0.035
Streptococcus	-0.050	*Intestinimonas*	0.032
Porphyromonas	-0.049	*Anaerotruncus*	0.031
Porphyromonas	-0.049	*Bacteroides*	0.029
Facklamia	-0.047	*Blautia*	0.029
Dialister	-0.043	*Barnesiella*	0.029
Alloscardovia	-0.043	*Bacteroides*	0.028
Anaerococcus	-0.041	*Blautia*	0.027
Prevotella	-0.040	*Pseudobutyrivibrio*	0.027
Peptoniphilus	-0.039	*Bacteroides*	0.025
Dialister	-0.033	uncultured Lachnospiraceae	0.025
Howardella	-0.031	uncultured Ruminococcaceae	0.025
Roseburia	-0.022	uncultured Defluviitaleaceae	0.024
Porphyromonas	-0.015	*Bifidobacterium*	0.024
Prevotella	-0.014	*uncultured* Ruminococcaceae	0.022
Actinotignum	-0.012	*uncultured* Lachnospiraceae	0.022
Actinotignum	-0.006	*Bacteroides*	0.013
Fusobacterium	-0.004		

Table 2 shows differences in genera estimated with the MaAsLin analysis. It shows genera that corresponds with OTUs that significantly differed ($p < 0.05$) after antimicrobial drug use. Antimicrobial drug use was analyzed as follows: no use (0), use >96 months before sampling (1), use 73-96 months before sampling (2), use 49-72 months before sampling (3), use 25-48 months before sampling (4), use 13-24 months before sampling (5), use 0-12 months before sampling (6). The estimate is a measure of the strength of the association where negative estimates mean that the OTU is lower in users of antimicrobial drugs, whereas the positive OTUs are higher in users of antimicrobial drugs. Duplicate genera refer to different OTUs of the same genus.

had the strongest influence on the genitourinary microbiota. However, it could be assumed that adjustment for the number of prior drug prescriptions did not influence the results.

Due to this and due to the cross-sectional study design, our results do not differentiate between antimicrobial treatment as a cause for dysbiosis versus the possibility that long-term dysbiosis was the cause of UTIs and subsequent antimicrobial treatment. This must be considered, since it has been hypothesized that UTIs are the result of dysbiosis of the microbiota in the genitourinary tract.[16] There are several arguments in favor of the hypothesis that dysbiosis of the genitourinary microbiota has caused UTIs, resulting in antimicrobial treatment. First, several of the antimicrobial drugs groups that have been prescribed to our participants, e.g. sulfonamides and trimethoprim (J01E) and nitrofuran derivatives (J01XE) are mainly, if not solely, prescribed for urinary tract infections by general practitioners (GPs) in the Netherlands. Second, in our population the dysbiosis was persistent for years after stopping the antimicrobial drugs. Although, this could also mean that antimicrobial drugs can have a persistent effect on the genitourinary microbiota.

A few other studies investigated the effects of antimicrobial drugs on the genitourinary microbiota or the effect of a specific composition of the genitourinary microbiota on UTIs. Differences were demonstrated in the urinary microbiota of kidney transplant patients who received prophylactic trimethoprim-sulfamethoxazole treatment compared to healthy controls, indicating that the genitourinary microbiota may be modified by antimicrobial drugs use.[17] In contrast to our study, the genitourinary microbiota of patients using trimethoprim-sulfamethoxazole had a decreased microbial diversity compared to healthy controls. This may be due to current versus past antimicrobial drug use, age and use of immunosuppressive medication of the kidney transplant patients, and the fact that this group differs from community-dwelling elderly.[17] Another study has already shown associations between the urinary microbiota and UTIs. Differences were shown in the microbiota of women on the day of surgery between women who did or did not develop a post-operative UTI.[18] However, one might also argue that changes in microbiota caused by antimicrobial drugs increase susceptibility to UTIs. For instance, it was shown in a cohort with 113 women that 27% experienced at least one recurrence within 6 months after an initial UTI, whereas in a cohort of 179 Finnish women 44% had recurrences.[19,20] Also, it was shown in mice that transient exposure to *Gardnerella vaginalis*, a member of the vaginal microbiota, can trigger *E.coli* reservoirs in the bladder to cause a UTI,[21] which might be an effect of antibiotic use. In our population, *Lactobacillus*, which is thought to play a role in the prevention of UTIs in women[22] was lower in the participants who used antimicrobial drugs. Also, a depletion of *Lactobacillus iners* in urine has recently been associated with postoperative UTI risk. This study also showed that enrichment of a diverse mixture of uropathogens was associated with postoperative UTI.[23] We found that *E.coli* was higher in the participants who had used antimicrobial drugs. It is not clear what the cause is or the consequence, and therefore further studies to elucidate the causal relationship between the genitourinary microbiota and the use of antimicrobial drugs are needed.

The strength of our study is that community-dwelling participants from The Rotterdam Study were included. The Rotterdam Study has prospectively gathered records without prior knowledge of research hypotheses. This includes data on drug prescriptions obtained from a collaborative database of all community pharmacies in the Ommoord area. Furthermore, the performed analyses compared the total microbiota compositions instead of comparing individual elements separately. However, our study also has some limitations. First of all, we only had a small sample size, but even in these small groups we could detect significant differences in microbiota. A second possible limitation may be that all participants were 70 years or older, whereas it has been shown that the diversity of gut microbiota declines after the age of 70,[24] and it has been assumed that the urinary microbiota also change with age.[7,25] Although, the genera found in the genitourinary microbiota in our study were also found by others, our findings should be extrapolated with care to younger individuals, especially premenopausal women.[7] A third limitation is the methods that we used. We collected midstream urine samples compared to urinary catheterization used in some other studies. Unfortunately, the latter is difficult to accomplish in a community-dwelling cohort of healthy elderly. The participants obtained clear instructions for collecting clean-catch midstream urine and diverse collection methods have indicated that the urinary microbiota is not simply the consequence of contamination or urethral colonization.[7,17] However, it should be kept in mind that it has been shown that the microbiota from voided urine contains a mixture of urinary and genital tract bacteria and therefore we called it the genitourinary microbiota.[26] Additionally, we used centrifugation to precipitate the bacteria to obtain enough DNA for analysis, but this could have introduced bias, since centrifugation will enrich for bacteria that pellet well. In this study, we excluded three participants from the analyses, due to too little bacterial DNA being present in their sample. This has also occurred in another study (3 out of 16),[7] indicating that it is not always possible to obtain sufficient DNA from urine with the present techniques.

In conclusion, we have shown that the composition of the genitourinary microbiota is associated with the use of antimicrobial drugs. It is not clear whether genitourinary dysbiosis predisposes for UTI with subsequent antibiotic treatment or that the antibiotic use causes the dysbiosis. Further studies are needed to elucidate the causal relationship between the composition of the genitourinary microbiota, UTIs and the use of antimicrobial drugs.

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Chapter 4.2

Long-term Effects of Antimicrobial Drugs on the Composition of the Human Gut Microbiota.

Marlies Mulder, Djawad Radjabzadeh, Jessica Kiefte-de Jong, Andre Uitterlinden, Robert Kraaij, Bruno Stricker, Annelies Verbon

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Abstract

Objective Antimicrobial drugs are known to have effects on the human gut microbiota. We studied the long-term temporal relationship between several antimicrobial drug groups and the composition of the human gut microbiota determined in feces samples

Methods Feces samples were obtained from a community-dwelling cohort of middle-aged and elderly individuals (Rotterdam Study). Bacterial DNA was isolated and sequenced using V3/V4 16 S ribosomal RNA sequencing (Illumina MiSeq). The time between the last prescription of several antimicrobial drug groups and the day of sampling was categorized into 0–12, 12–24, 24–48 and >48 months. The effects of the antimicrobial drug groups on the Shannon alpha-diversity (diversity), the Bray–Curtis beta-diversity (community structure), the Firmicutes/Bacteroidetes (F/B) ratio and individual genera were determined.

Results We studied the gut microbiota of 1413 individuals (57.5% female, median age 62.6 years). The alpha-diversity was significantly lower up to 4 years after prescriptions of macrolides and lincosamides. It was also lower in the first year after the use of beta-lactams. The community structure (beta-diversity) of the microbiota was significantly different up to 4 years for macrolides and lincosamides, the first year for beta-lactams and at least the first year for quinolones. For the F/B ratio, drugs with a high anaerobic activity shifted the ratio toward Firmicutes in the first year whereas other antimicrobial drugs shifted the ratio toward Bacteroidetes.

Conclusions Use of antimicrobial drugs is associated with a shift in the composition of the gut microbiota. These effects differ in strength and duration, depending on the antimicrobial drug group used. These findings should be considered when prescribing antimicrobial drugs.

Introduction

The gut microbiota plays a role in a variety of processes, such as protection against overgrowth of pathogenic micro-organisms, in the development of the host immune response, in neurologic signaling and in the synthesis and metabolism of several compounds, such as short-chain fatty acids (SCFAs).[1,2] In particular, the SCFA butyrate is said to have an important function in the maintenance of a healthy colonic epithelium.[3]

The composition of the gut microbiota may differ with age,[4] gender and BMI[5] and can change under the influence of diet,[6,7] physical activity,[8] diabetes[9] and use of drugs, such as proton pump inhibitors,[10] corticosteroids[11] and statins.[12] Furthermore, it is known that it can be influenced by the use of antimicrobial drugs (post-antibiotic dysbiosis). The use of antimicrobial drugs has been reported to increase the vulnerability to overgrowth of potentially pathogenic bacteria, such as *Clostridium difficile*, with the risk of pseudomembranous colitis. Moreover, it has been described to cause a loss of diversity of the gut microbiota, cause a decrease of important taxa, alter gene expression, select for intrinsically resistant bacteria, and select for new mutations.[13-15] Additionally, dysbiosis has also been designated as a factor that promotes horizontal gene transfer, thereby increasing the probability of spreading antibiotic resistance genes.[16]

In adults, some information is available about the effects of specific antimicrobial drug groups on the composition of the gut microbiota. Short-term exposure to clindamycin was shown to cause a shift of the gut microbiota, for example a decline in the diversity of Bacteroidetes.[17] Furthermore, *Lachnospiraceae* abundancy in the gut was decreased up to 6 months after the use of amoxicillin or azithromycin.[18] Also, one study showed the effects of using beta-lactam antibiotics in the 12 months before sampling in a population-based cohort.[19] However, most studies have investigated these effects in small populations, studying rather short-term effects and using a variety of methods to investigate the microbiota. Therefore, the objective of this study was to describe and compare the effects and the duration of the effects of different antimicrobial drug groups on the composition of the human microbiota in feces samples from a large population of community-dwelling middle-aged and elderly individuals using different outcomes that characterize the microbiota.

Patients and methods

Source population

The feces samples that were used in this study were obtained from study participants of the third cohort (RSIII) of The Rotterdam Study (RS), a prospective population-based study. This

cohort includes 3122 individuals, who were recruited in the period March 2012 to June 2014 and who were 45 years and older, living in the Ommoord district in Rotterdam. All participants are invited every 3–4 years for follow-up interviews and examinations. More detailed information on the Rotterdam Study can be found elsewhere.[32]

Gut microbiota composition

Stool samples were collected at home by the participants using a Commode Specimen Collection System (Covidien, Mansfield, MA). An aliquot of approximately 1 g was transferred to a 25 × 76 mm feces collection tube (Minigrip Nederland, Lelystad, The Netherlands) and sent through regular mail to the Erasmus MC. A short questionnaire addressing amongst others date and time of defecation was filled out by the participants (response percentage 69%). After receipt, the samples were stored at −20°C. Approximately, 300 mg of feces was homogenized in stool stabilizing buffer. Automated DNA-isolation (Arrow DNA; DiaSorin S.p.A., Saluggia, Italy) was performed using the Arrow DNA kit according to the manufacturer's instructions and included bead-beating in Lysing Matrix B tubes containing 0.1 mm silica beads (MP Biomedicals, LLC, Bio Connect Life Sciences, Huissen, The Netherlands). The hypervariable regions V3 and V4 of the (bacterial) 16 S rRNA gene were amplified and sequenced using the Illumina MiSeq 2 × 300 base pairs protocol (FADROSH, PMID: 24558975). Phylogenetic multi-sample profiling was performed using an in-house developed pipeline based on the QIIME 1.9.0 (Caporaso PMID: 20383131) and USEARCH version 8.1 (Edgar PMID: 23955772) software packages. After subsampling at 10,000 reads per sample, taxonomy was assigned using the naïve Bayesian RDP classifier (vs 2.12)[33] and the SILVA database (v128; Quast PMID: 23193283). The OTU table was cleaned by filtering out low abundance OTUs (<0.005% of total reads per OTUs and OTUs present in <1% of the samples). Samples with unknown information of time in the mail, samples arriving 3 days after collection and samples from participants who used antibiotics during or just before sampling were removed.

Medication use

The date of the last prescription of an antimicrobial drug before feces sampling was obtained from a collaborative database of all community pharmacies in the Ommoord area that goes back to 1 January 1995. The antimicrobial drug prescriptions were grouped on Anatomical Therapeutic Chemical (ATC) code, which included: tetracyclines (J01A), amphenicols (J01B), beta-lactam antibacterials (J01C), other beta-lactam antibacterials (J01D) (which includes all generations of cephalosporins and carbapenems), sulfonamides and trimethoprim (J01E), macrolides and lincosamides (J01F) (J01F also includes streptogramins, but these were not prescribed in the study period), aminoglycoside antibacterials (J01G), quinolone antibacterials (J01M), glycopeptide antibacterials (J01XA), polymyxins (J01XB), steroid antibacterials (J01XC), imidazole derivatives (J01XD), nitrofuran derivatives (J01XE) and other antibacterials (J01XX). For each antimicrobial drug group, the time interval between the date of the last prescription and

feces sampling was calculated and categorized into the use of 0–12, 12–24, 24–48 and >48 months before sampling or no use of the antimicrobial drug group. Additionally, the antimicrobial drugs were classified in a group antimicrobial drugs with a high activity against anaerobic species (anaerobic+), consisting of combinations of penicillins, including beta-lactamase inhibitors (J01CR), lincosamides (J01FF) and imidazole derivatives (metronidazole) (J01XD) and a group without this activity (anaerobic-) (all other antimicrobial drugs).

Confounders

The following potential confounders (at the time of feces sampling) were taken into account in the analyses: age, sex, BMI, diabetes (use of anti-diabetic drugs (A10)), use of co-medication, time in the mail of the feces sample and batch number representing two batches of DNA isolation: the first 102 DNA isolation runs with a relatively high yield were labeled 0 and the last 32 runs with a relatively low yield were labeled 1. Patients who were prescribed a drug within 90 days before feces sampling were considered as current user of that drug. Drugs that possibly influence the composition of the microbiota according to literature: proton pump inhibitors (A02BC),[10] statins (C10AA),[12] systemic corticosteroids (H02),[11] antipsychotics (N05A),[34] selective serotonin reuptake inhibitors (SSRIs) (N06AB),[35] antineoplastic agents (L01),[36] and tacrolimus (L04AD).[37] Since proton pump inhibitors may have been sold over the counter, participants were also asked if they used proton pump inhibitors.

Other potential confounders for studying the association with the gut microbiota are diet and smoking. Adjustment for diet was performed by adjusting for the dietary guidelines score (DGS), which is a score that varies from 0 to 14 and represents the adherence to the Dutch dietary guidelines which include 14 items: vegetables (≥200 g/day), fruit (≥200 g/day), whole-grains (≥90 g/day), legumes (≥135 g/week), nuts (≥15 g/day), dairy (≥350 g/day), fish (≥100 g/week), tea (≥450 mL/day), ratio whole-grains:total grains (≥50%), ratio unsaturated fats and oils:total fats (≥50%), red and processed meat (<300 g/week), sugar-containing beverages (≤150 mL/day), alcohol (≤10 g/day) and salt (≤6 g/day).[38] Adjustment for smoking was performed by adjusting for the smoking status (never, ever, current).

Statistical analyses

We performed several analyses in order to study the association between antimicrobial drug use and the composition of the gut microbiota, using several measures described below. For the diversity, Firmicutes/Bacteroidetes ratio (F/B ratio) and community structure analysis, we performed two models. In model 1, we adjusted for the above-mentioned confounders (sex, age, BMI, diabetes, use of co-medication, time in the mail and batch number). In model 2, we additionally adjusted for the categorized use of other antimicrobial drug groups (thus, for example: the association between tetracyclines and the gut microbiota was adjusted for the mentioned confounders and for categorized use of beta-lactam antibacterials, categorized use of sulfonamides and trimethoprim, categorized use of macrolides and lincosamides, categorized

use of quinolones, and categorized use of nitrofuran derivatives). For diet and smoking, the data were not available for all participants: 269 (19.0%) were missing for diet and 108 (7.6%) for smoking. Therefore, adjustment for these confounders was only performed in two sensitivity analyses.

Diversity analysis

The Shannon index was used to calculate the alpha-diversity (measure of diversity of species within a sample). In order to obtain a normal distribution (according to the Kolgomorov–Smirnov test), it was transformed by calculating the cube. For each antimicrobial drug group, a linear regression was performed with the transformed Shannon alpha-diversity as the dependent variable, and four dummies for antimicrobial drug use of the specific group and the confounding variables as independent variables. The outcome was back transformed for male gender, batch 0, with median age, median BMI, for those who had no diabetes, who used no co-medication, of whom the sample was a median time in the mail and who used no other antimicrobial drugs than the one of interest (= average person). *P*-values <0.05 were considered to be significant.

The Firmicutes/Bacteroidetes ratio

The F/B ratio was calculated and logarithmically transformed to obtain a normal distribution. Different linear regressions were performed and transformed back to the average person as described above but now with as a dependent variable the transformed F/B ratio. *P*-values <0.05 were considered to be statistically significant.

Community structure analysis

MiRKAT is a recently developed package, available in the statistical program R, that tests for associations between microbiota composition and an outcome, using a semi-parametric kernel machine regression.[39] MiRKAT (using 100,000 permutations) was used to investigate differences in the composition of the fecal microbiota using the Bray–Curtis beta-diversity distance (measure of dissimilarity of species composition between sample pairs). *P*-values <0.05 were considered to be statistically significant.

Single genera analyses

The genera that were significantly different in individuals who had used antimicrobial drugs were determined using the MaAsLin (Multivariate Association with Linear Models) function.[40] The categorized variable for the use of each antimicrobial drug group was linearly included in the model (0 for no prescription at all, 4 for latest prescription in 0–12 months before sampling). For the analysis, the default settings were used: a false discovery rate of 25% and q-values <0.05 were considered to be statistically significant.

Results

We obtained a dataset with microbiota data from 1427 participants, of whom 14 (1.0%) were excluded, because no pharmacy data were available. From the remaining 1413 participants, 812 (57.5%) were female and 601 (42.5%) were male with a median age of 62.6 years (IQR 58.6–66.1), a median BMI of 26.8 (IQR 24.5–29.7) and the feces sample had been in the mail for a median time of 1 day (IQR 1–2 days). Furthermore, 323 individuals used proton pump inhibitors and 252 used a statin. There was no use of tacrolimus and the use of antineoplastic agents (3 participants, 0.2%) was very low; therefore, these drugs were not included in the models. A total of 1281 (90.6%) participants had received at least 1 prescription of an antimicrobial drug during follow-up (at least 17 years). Most participants (73.7%) had 1 or more prescriptions of beta-lactam antibiotics, other frequently used antibacterial drugs were tetracyclines (57.7%) and macrolides and lincosamides (44.0%). The number of prescriptions for amphenicols (J01B), other beta-lactam antibacterials (J01D), aminoglycoside antibacterials (J01G), glycopeptide antibacterials (J01XA), polymyxins (J01XB), steroid antibacterials (J01XC), imidazole derivatives (J01XD) and other antibacterials (J01XX) was very low and these groups could not be analyzed separately (**Table 1**). The correlations between the different antimicrobial drug groups were low.

Shannon alpha-diversity

The median overall diversity was 4.10 (IQR 3.73–4.37). The strongest and most prolonged effect on diversity was seen in the group of the macrolides and lincosamides (J01F). Transforming the beta's back to physiological values (for an average person) resulted in a significantly lower diversity of 0.48 for 0–12 months after use of macrolides and lincosamides (J01F); a lower diversity of 0.28 (which was not significant when adjusting for all other antimicrobial drug use) for 12–24 months after use, a significantly lower diversity of 0.35 for 24–48 months after use and a significantly lower diversity of 0.17 for 48 months or longer after use. We also showed a significantly lower diversity of 0.24 after the use of beta-lactam antibacterials (J01C) within 1 year before feces sampling. No change in diversity was seen after the use of tetracyclines (J01A), sulfonamides and trimethoprim (J01E), quinolones (J01M) and nitrofurans (J01XE). (untransformed beta's of model 2 in **Figure 1**)

We also classified the antimicrobial drugs in antimicrobial drugs with anaerobic activity (consisting of combinations of penicillins, including beta-lactamase inhibitors (J01CR), lincosamides (J01 FF) and imidazole derivatives (metronidazole) (J01XD): anaerobic+) and a group without this activity (all other antimicrobial drugs: anaerobic-). The use of anaerobic+ antimicrobial drugs was associated with a stronger and more prolonged effect on diversity than the use of antimicrobials without this activity. For an average person, diversity after the use of anaerobic+ antimicrobial drugs was 0.51 lower for the 0–12 months period and 0.36 lower for

Table 1: Use of antimicrobial drugs in the study population

Antimicrobial drug group	0-12 months*	12-24 months*	24-48 months*	> 48 months*	None
Antibacterial for systemic use (J01)	355	217	236	473	132
Tetracyclines (J01A)	100	103	152	461	597
Amphenicols (J01B)	0	0	0	0	1413
Beta-lactam antibacterials (J01C)	179	131	163	569	371
Other beta-lactam antibacterials (J01D)	1	2	1	11	1398
Sulfonamides and trimethoprim (J01E)	20	23	55	267	1048
Macrolides, lincosamides and streptogramins (J01F)	62	49	106	405	791
Aminoglycoside antibacterials (J01G)	1	0	0	1	1411
Quinolone antibacterials (J01M)	35	38	48	154	1138
Glycopeptide antibacterials (J01XA)	0	0	0	1	1412
Polymyxins (J01XB)	0	0	0	0	1413
Steroid antibacterials (J01XC)	0	0	0	0	1413
Imidazole derivatives (J01XD)	0	0	0	2	1411
Nitrofuran derivatives (J01XE)	76	38	67	121	1111
Other antibacterials (J01XX)	13	3	5	3	1389

Use of antibacterial drugs per group and overall for each time period of prescription to fecal sampling (0–12, 12–24, 24–48, >48 months, or no use). The use of amphenicols (J01B), other beta-lactam antibacterials (J01D), aminoglycoside antibacterials (J01G), glycopeptide antibacterials (J01XA), polymyxins (J01XB), steroid antibacterials (J01XC), imidazole derivatives (J01XD) and other antibacterials (J01XX) is too low to analyze further. *Time period between prescription of antimicrobial drug and fecal sampling.

the 12–24 months period. Diversity was only 0.23 lower (for an average person) 0–12 months after the use of anaerobic-antimicrobial drugs. (untransformed beta's in **Figure 2**) We also performed two sensitivity analyses with additional adjustment for diet and smoking, which slightly shifted the use of beta-lactams in the first year before sampling, resulting in a not significant difference.

Firmicutes/Bacteroidetes ratio

The median F/B ratio was 0.085 (IQR 0.037–0.21). We could not show any significant differences for any of the different antimicrobial drug groups on the F/B ratio, both in model 1 and in model 2, in which we additionally adjusted for all antimicrobial drug use. (untransformed beta's for model 2 in Figure S3). However, the F/B ratio significantly shifted toward Firmicutes in the 0–12 months before sampling after the use of anaerobic+ antimicrobial drugs. Furthermore, a significant shift could be demonstrated 12–24 months, 24–48 months and 48 months after the use of anaerobic- antimicrobial drugs toward Bacteroidetes of respectively 0.18, 0.20 and 0.16. (untransformed beta's in **Figure 3**).

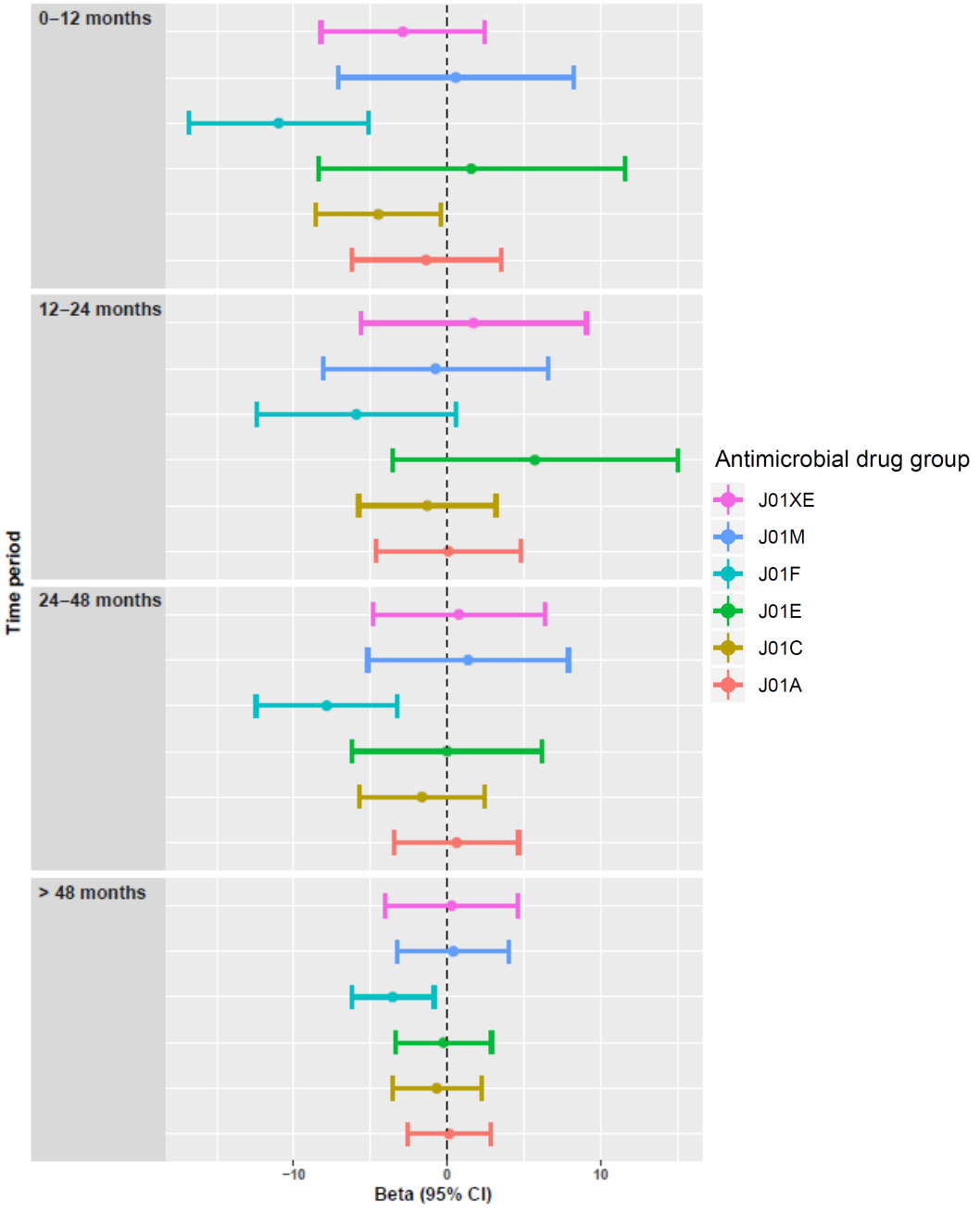

Figure 1: Diversity after antimicrobial drug use. Plots of the beta's with 95% confidence intervals of the linear regression with as dependent variable the transformed (cube) Shannon alpha-diversity and as independent variables the different antimicrobial drug groups. All antimicrobial drug groups were analyzed with dummy variables with categories of use of 0–12, 12–24, 24–48 and >48 months compared to no use before sampling. The analyses were adjusted for age, sex, BMI, diabetes, time in mail, batch number, use of statins, PPIs, SSRIs, antipsychotics and systemic corticosteroids and (categorized) use of all other antimicrobial drugs.

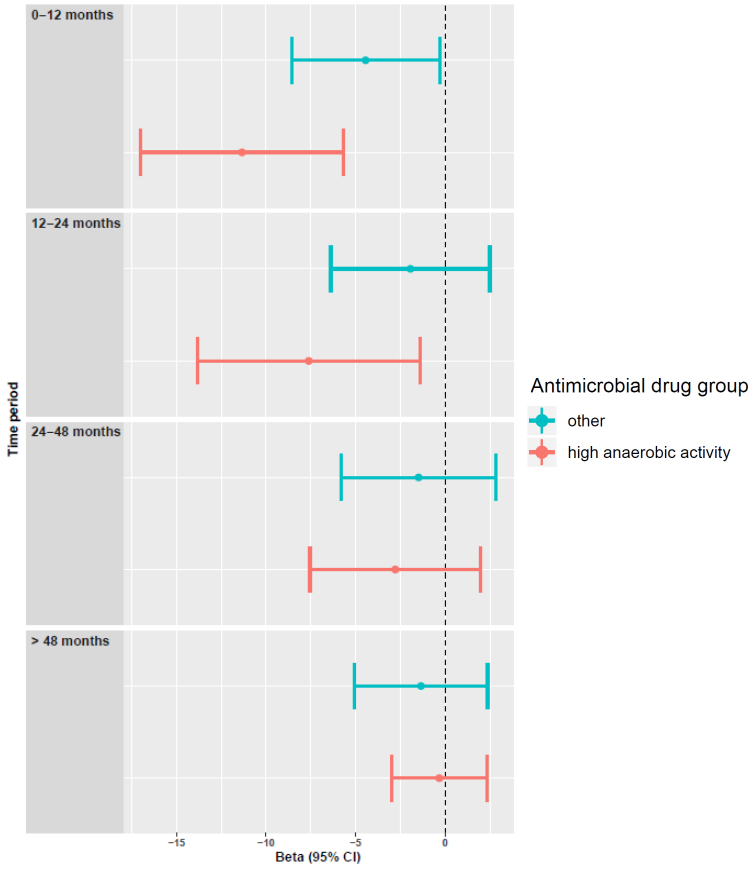

Figure 2: Diversity after using antimicrobial drugs with high anaerobic activity vs other antimicrobial drugs. Plots of the beta's with 95% confidence intervals of the linear regression with as dependent variable the transformed (cube) Shannon alpha-diversity and as independent variables the combined antimicrobial drugs that have a strong anaerobic activity (anaerobic + drugs) and the combined remaining antimicrobial drugs (anaerobic- drugs). Both were analyzed with dummy variables with categories of 0–12, 12–24, 24–48 and >48 months compared to no use. The analyses were adjusted for age, sex, BMI, diabetes, time in mail, batch number, use of statins, PPIs, SSRIs, antipsychotics and systemic corticosteroids and (categorized) use of all other antimicrobial drugs.

Community structure

Concerning the community structure (beta-diversity), we again found significant differences for macrolides and lincosamides (J01F) in all time categories. We also found a difference for beta-lactams (J01C) in 0–12 months before sampling. Finally, we found significant differences for quinolones both for 0–12 and 24–48 months before sampling, but not for 12–24 months (**Figure 4**). Most and strongest differences in genera were seen after the use of macrolides and lincosamides.

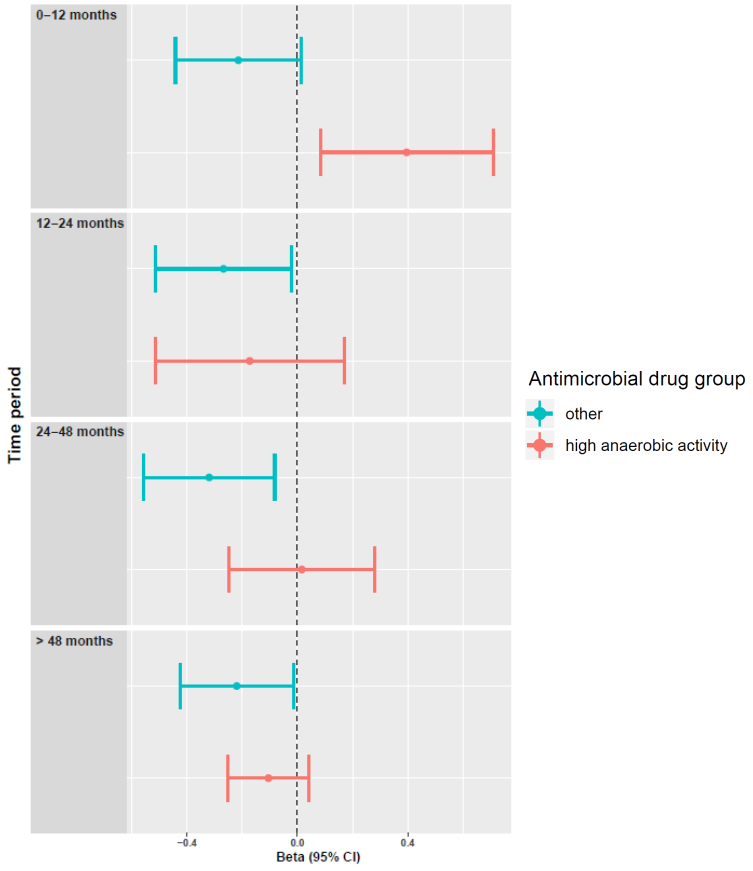

Figure 3. Firmicutes/Bacteroidetes ratio after using antimicrobial drugs with high anaerobic activity vs other antimicrobial drugs. Forest plots of the relative risks with 95% confidence intervals of the linear regression with as dependent variable the transformed (logarithmic) Firmicutes/Bacteroidetes ratio and as independent variables the combined antimicrobial drugs that have a strong anaerobic activity and the combined remaining antimicrobial drugs ("other"). Both were analyzed with dummy variables with categories of 0–12, 12–24, 24–48 and >48 months compared to no use. The analyses were adjusted for age, sex, BMI, diabetes, time in mail, batch number, use of statins, PPIs, SSRIs, antipsychotics and systemic corticosteroids and (categorized) use of all other antimicrobial drugs. A positive beta indicates a shift toward Firmicutes, whereas a negative beta indicates a shift toward Bacteroidetes.

Discussion

In this study, we showed an association between the use of different oral antimicrobial drugs and the diversity of the gut microbiota in feces samples of a middle-aged and elderly community-dwelling population. The strongest and most prolonged effects on the microbiota diversity were shown for macrolides and lincosamides, and these effects lasted up to several years after use.

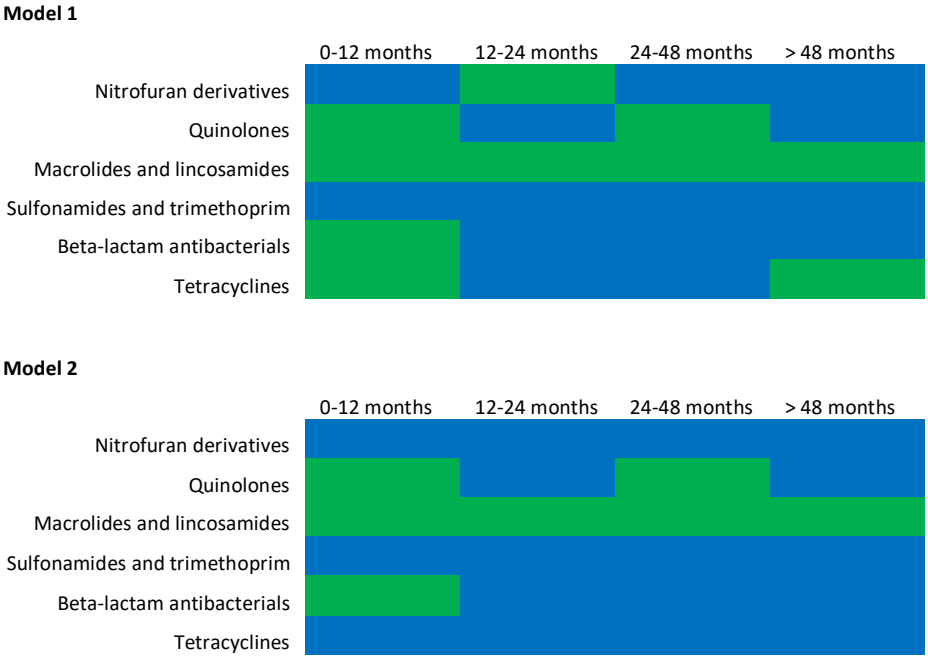

Figure 4. Effects of antimicrobial drug use on community structure. Significance table for all antimicrobial drug groups for all time categories studied in model 1, thus adjusted for age, sex, BMI, diabetes, time in mail, batch number, use of statins, PPIs, SSRIs, antipsychotics and systemic corticosteroids. (Top) Significance table for all antimicrobial drug groups for all time categories studied in model 2, thus adjusted for age, sex, BMI, diabetes, time in mail, batch number, use of statins, PPIs, SSRIs, antipsychotics and systemic corticosteroids and (categorized) use of all other antimicrobial drugs. (Bottom) In these significance tables **green** indicates significant ($p < 0.05$), **blue** indicates not significant.

Also, diversity was lower and community structure was different in the first year after beta-lactam use. Furthermore, the use of antimicrobial drugs with a high anaerobic activity was associated with a shift toward Firmicutes. This, in contrary to the use of antimicrobial drugs without this activity, which resulted in s shift toward Bacteroidetes.

Increasing evidence shows that changes in the microbiota by antimicrobial drugs are associated with a variety of diseases. A study in Finnish school children reported an association between frequent macrolide use in early life (<2 years) and the development of asthma.[20] Furthermore, the use of several antimicrobial drug groups was shown to be associated with several cancers in a large-nested case–control study, possibly acting via the gut microbiota.[21] Also, but still unproven, it has been suggested to prescribe probiotics simultaneously with antimicrobial drugs. A large systemic review showed that there is evidence that probiotics are effective in

preventing *Clostridium difficile*-associated diarrhea in not-immunocompromised individuals with a high baseline risk.[22] Furthermore, in mice concurrent probiotics treatment during or after antibiotic therapy caused suppression of *Enterobacteriaceae* outgrowth, while promoting blooming of Firmicutes.[23] Therefore, the influence on the gut microbiota should be taken into consideration when prescribing antibiotic drugs.

Many studies investigated the microbiota with different outcome parameters, included few participants or reported only short-term effects.[17-19] A strength of our study is that we studied the gut microbiota in a large population-based cohort with detailed information on antimicrobial drug prescriptions and over more than a 4-year time period using different outcome parameters. Furthermore, we adjusted our models for several potential confounders, such as sex, age, BMI, diabetes and use of co-medication (statins, systemic corticosteroids, proton pump inhibitors, SSRIs and antipsychotics). Diversity is the most frequently reported outcome in microbiota studies and loss of diversity appears as the most consistent finding of intestinal dysbiosis.[24] We also report on the Firmicutes/Bacteroidetes ratio, and the community structure, using the beta-diversity. The different outcome parameters enable a broader interpretation of the effect of antibiotic use on gut microbiota. Unfortunately, we could not compare the microbiota after with the microbiota before antimicrobial drug use, but the time frame, the cohort size and the fact that the participants were prescribed antibiotics by their physician for an infection and not specifically for this study made such a study design not feasible. Also, because of the length of the study time, other factors, such as intestinal surgery, could also have influenced the microbiota. However, because of the size of the cohort, we assume that the effect of these factors is small. Another limitation is that the feces was sent to the study center by the participants via mail, which might have influenced changes of the microbiota composition by environmental factors, such as temperature. However, the effects of our collection method have been studied and has resulted in the exclusion of samples that were in the mail longer than three days. Furthermore, the time in the mail was included as a covariate in the analyses. Also, the microbiota within our cohort had similar profiles as those in two other large population-based cohorts.[25] Another limitation may be that our results were obtained from feces samples and may not reflect the microbiota more proximal in the digestive tract.[26] Furthermore, we only used the last prescription before sampling, not taking into account the antimicrobial drug prescriptions used previously. However, we showed that correlations between the use of antimicrobial drugs of different classes were low and additionally, we adjusted for all other antimicrobial drug use.

All our results pointed to macrolides and lincosamides as the antimicrobial drugs with the highest ability to cause changes in the composition of the gut microbiota. Of these two types of antibiotics, lincosamides such as clindamycin probably have the strongest effect, since we also showed that antibiotics with a high anaerobic activity (which included clindamycin) had strong associations with the diversity of the microbiota. Another study has also shown long-lasting effects of clindamycin on the gut microbiota but only up to 2 years.[17] Furthermore, the macrolide

azithromycin was shown to have effects up to 6 months,[18] and a shift of the gut microbiota at phylum level was found in Finnish children of 2–7 years old after macrolide use in the 2 years before sampling.[20] Our data indicate that a shift in the composition of the gut microbiota persists for a longer time period.

Beta-lactam antibiotics caused a lower diversity and differences in community structure in the first year after use. Beta-lactams have been associated with effects on the composition of the gut microbiota in several small studies.[17] Use of tetracyclines has been associated with a relative increase in the abundance of Bacteroidetes.[27] Doxycycline use was shown to be associated with a lower diversity and a relative increase of Bacteroidetes in mice.[28] Nitrofurantoin was shown to have only minor effects in a study in patients with urinary tract infections.[29] These studies, however, investigated the effects after a maximum of a few months. Since we did not find any effects in our longer time periods, this might suggest that the effects of these antimicrobial drugs on the gut microbiota are restored after a few months. We also did not find any effects for sulfonamides and trimethoprim, but the use of these drugs (singly or in combination products) was very low.

Although we could not find an effect on the F/B ratio for separate antimicrobial drug classes, we found that the use of antimicrobial drugs with a high anaerobic activity was associated with a shift toward Firmicutes in the first year. In contrast, the use of antimicrobial drugs without this anaerobic activity was associated with a shift toward Bacteroidetes up to several years. Others also described effects on this ratio, but only directly after treatment, showing relatively more Bacteroidetes after the use of antimicrobial drugs.[30,31]

In conclusion, we showed that antimicrobial drugs, especially macrolides and lincosamides, are associated with a long-lasting shift in the gut microbiota. Further research is needed to explore the interaction and effect of specific antibiotics on the gut microbiota, considering the consequences of the use of antimicrobial drugs on the gut microbiota.

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Chapter 4.3

Prevalence of and Risk Factors for Extended-Spectrum Beta-Lactamase Genes Carriership in a Population-Based Cohort of Middle-Aged and Elderly

Marlies Mulder, Pascal Arp, Jessica Kiefte – de jong, Andre Uitterlinden, Corné Klaassen, Robert Kraaij, Wil Goessens, Annelies Verbon, Bruno Stricker

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Abstract

Objective Increasing worldwide resistance to beta-lactam antibiotics is an alarming development worldwide. Fecal carriership of TEM, SHV, CTX-M and CMY was studied in a community-dwelling population of middle-aged and elderly individuals.

Methods Feces was obtained from individuals of the Rotterdam Study. Carriership of the TEM, SHV, CTX-M and CMY genes was determined using real-time PCR (qPCR). Possible associations were investigated between carriership of these genes and several risk factors, such as the use of antimicrobial drugs, diabetes mellitus, proton pump inhibitor (PPI) use, travelling, the composition of the gut microbiota and intake of certain foods.

Results The most prevalent gene was TEM (53.0%), followed by SHV (18.4%), CTX-M (5.4%) and CMY (3.6%). Use of penicillins with extended spectrum was associated with TEM carriership, whereas use of macrolides and lincosamides was associated with TEM and SHV carriership. Interestingly, use of PPIs was associated with a higher prevalence of carriership of TEM, SHV and CMY (TEM: OR 1.34; 95%CI 1.05 – 1.77; SHV: OR 2.17; 95%CI 1.55 – 2.87; CMY: OR 2.26; 95%CI 1.23 – 4.11). Furthermore, associations were found between the richness and composition of the gut microbiota and TEM and SHV carriership.

Conclusions The prevalence of carriership of TEM was substantial, but the prevalence of carriership of the extended-spectrum β-lactamase gene, CTX-M and the AmpC β-lactamase gene, CMY was relatively low in this community-dwelling, population-based cohort. The composition of the microbiota might play a role in the retention of resistance genes, but future studies are necessary to further elucidate this relationship.

Introduction

Antimicrobial resistance (AMR) is increasing worldwide and is associated with high morbidity and mortality.[1] One of the alarming developments in AMR is the increasing resistance to beta-lactam antibiotics,[2, 3] which are important in the first-line treatment of a variety of infections. Beta-lactamases (BLs) are enzymes that inactivates penicillins, cephalosporins and, in some cases, carbapenem antibiotics. These antimicrobial drugs all have a beta-lactam ring, which can be hydrolyzed by BLs, thus inactivating the antimicrobial drug.

There are several important BL-families. The first discovered and most prevalent (up to 90% in *Escherichia coli*) plasmid-mediated BL in Gram-negative bacteria is TEM-1, which was discovered in the 1960s and only has activity towards penicillins and first generation cephalosporines. In the subsequent decades, numerous other TEM derivatives were reported, many of which are extended-spectrum beta-lactamases (ESBLs), which can also inactivate third generation cephalosporins. Another BL-family is that of the SHV genes. The SHV-1 BL is common in *Klebsiella pneumoniae* as it is chromosomally encoded. Variants of the SHV-1 BL can be harbored on transmissible plasmids and increasingly display ESBL activity.[4]

More recently, CTX- M, another group of transferable plasmids, was discovered as a new family of ESBLs. These are not related to TEM and SHV, and originate from Kluyvera spp., which are rarely of clinical significance.[4 5] Also found were plasmid encoded AmpC beta-lactamases (AmpC), such as the CMY family, which hydrolyze third generation cephalosporins, and are not inhibited by clavulanic acid.[6]

The human gut microbiota is increasingly recognized as a reservoir for antimicrobial resistance genes (the resistome). Selective pressure by antimicrobial drugs may favor selection of resistant bacteria.[7] Also, individuals can become carriers of resistant bacteria via environmental factors, i.e. food, water, soil, animals and other humans.[7-13] This may be important as digestive tract colonization with ESBL-producing Enterobacteriales in patients on intensive care units has been shown to be associated with developing an infection caused by these resistant bacteria.[14] However, in a study with healthy travelers, infection with a colonizing *E.coli* was rare.[15]

The prevalence of small-spectrum and extended spectrum beta-lactamase genes have been reported in many populations, mostly using clinical cultures of patients, although some also investigated the prevalence in a healthy population.[16-19] These studies have provided important insights into antimicrobial resistance genes in the human gut. In the current study, fecal carriership of TEM, SHV, CTX-M and CMY was assessed using real-time PCR (qPCR) in a community-dwelling population of middle-aged and elderly individuals in Rotterdam, The Netherlands. Also studied was the association between carriership of these genes and the use of antimicrobial drugs, the composition of the gut microbiota and other potential risk factors.

Materials and methods

Source population

The Rotterdam Study was designed as a prospective population-based cohort study as described in detail elsewhere.[20] Briefly, all inhabitants, aged at least 55 years and living in the well-defined Ommoord district in Rotterdam, The Netherlands were invited to participate (response rate 78%) in 1991 (RSI). In 2000 (RSII) and 2006 (RSIII), two other cohorts were included with inhabitants at least 55 years, and at least 45 years, respectively. The total cohort now comprises 14,926 participants, with survivors invited every 3-4 years for follow-up interviews, physical examination, laboratory and functional tests, and imaging procedures.

Beta-lactamase genes

This was a cross-sectional study in which individuals of the second wave of the third cohort (RSIII-2) of the Rotterdam Study were asked to collect a feces sample and send it to the Erasmus MC by mail. The samples were then stored at -20°C. An aliquot of approximately 300 mg was homogenized in stool stabilizing buffer according to the manufacturer's protocol (Arrow Stool DNA; Isogen Life Science). Homogenized samples were bead-beated in Lysing Matrix B tubes containing 0.1 mm silica beads (MP Biomedicals) using the MagNA Lyser instrument (Roche Diagnostics) at 7,000 rpm for 45 seconds. Samples were then centrifuged at 6,000 g for 5 min and 0.5 ml of supernatant was subjected to automated DNA isolation (Arrow; DiaSorin S.P.A) according to the manufacturer's protocol using setting 'Stool DNA 2.0'. DNA concentrations were measured with picogreen (Invitrogen) and diluted to 20 ng/µL. A qPCR targeting the bacterial 16S gene [21] was used to determine the bacterial DNA quantity per sample.

The presence of CTX-M, SHV, TEM and CMY genes was determined using qPCR with primer and probe sequences from Roschanski et al.[22] For the detection of TEM (FAM-labelled Taqman probe) and SHV (VIC-labelled Taqman probe), singleplex reactions were performed containing 40 ng fecal DNA, 3 pmol forward and reverse primers and 3 pmol Taqman probe and 1x Type-it Fast SNP PCR Master Mix (Qiagen) in a reaction volume of 5 µL. For the detection of CMY (FAM-labelled Taqman probe) and CTX-M (VIC-labelled Taqman probe), a duplex reaction was performed containing 40 ng fecal DNA, 5 pmol forward and reverse primers, 5 pmol of each Taqman probe and 1x Type-it Fast SNP PCR Master Mix (Qiagen) in a reaction volume of 5 µL. Reactions were performed in a 7900HT Real time PCR machine (Applied Biosystems) with initial denaturation at 95°C for 5 minutes and 45 cycles at 95°C for 15 seconds and 60°C for 30 seconds. Amplification curves and Cq-value (cycle threshold) determination were done with SDS 2.3 software.

For SHV, CTX-M and CMY, all blank reactions did not show a Cq-value. However, some blank reactions showed a Cq-value for TEM because of the presence of contaminating microbial DNA in commercial Taq polymerase preparations. The distribution of Cq-values for TEM of 73 blank

reactions using water instead of DNA is shown in supplementary figure 1. For the statistical analyses described below, a cut-off was set at 31.3.

To validate the results, all samples that gave a Cq-value for SHV, CTX-M and CMY were repeated in a second run. For TEM a random selection of 8% of the samples were repeated in a second run to validate the results.

Determinants

Sociodemographic characteristics

General characteristics of the study population, including age, sex, BMI and socioeconomic status (SES), were collected at baseline and during the follow-up interviews. Diabetes mellitus was defined as use of hypoglycemic medication. Furthermore, the participants filled out a small questionnaire before sending in the fecal sample to determine whether they had been traveling abroad in the month before sampling or whether they had used probiotics in the 3 days before sampling.

Medication use

Data on medication use for each individual were obtained from all community pharmacies in the Ommoord area, consisting of all out-patient prescriptions since 1st January, 1995. The number of prescriptions (since 1995) and the date of the last prescription before sampling of several antimicrobial drug groups was obtained from these data and grouped on ATC code. This included: penicillins with extended spectrum (ATC-Code J01CA), beta-lactamase sensitive penicillins (J01CE), beta-lactamase resistant penicillins (J01CF), combinations of penicillins, including beta-lactamase inhibitors (J01CR), first-generation cephalosporins (J01DB), second-generation cephalosporins (J01DC) third-generation cephalosporins (J01DD), carbapenems (J01DH), but also tetracyclines (J01A), sulphonamides and trimethoprim (J01E), macrolides and lincosamides (J01F), quinolones (J01M) and nitrofuran derivatives (J01XE). The number of prescriptions since 1995 for each drug group was categorized into: no prescriptions; 1 prescription; 2 prescriptions; or >2 prescriptions. As there were fewer prescriptions for beta-lactamase sensitive penicillins and beta-lactamase resistant penicillins these were categorized into: no prescriptions; 1 prescription; and >1 prescription.

Proton pump inhibitors (PPIs) (A02BC) are sold over the counter in The Netherlands, therefore the use of these drugs was assessed as use within 90-days before sampling or noted according to the interview.

Diet and lifestyle

Habitual food intake of participants of several cohorts in the Rotterdam Study was determined (2006-2012) using an extensive semi-quantitative food frequency questionnaire (FFQ) with 389 items.[23, 24] Participants who reported an unusual energy intake of <500 kcal/day or >5000

kcal/day were excluded from the analyses. Dietary intake is prone to measurement error and confounding; therefore, intake of each food item was adjusted for total energy intake using the residual method as previously explained.[25] The energy-adjusted dietary variables were categorized into quartiles but added linearly to the multivariable model to obtain an odds ratio (OR) of the trend. To prevent false-positive results because of multiple comparisons, this study only investigated the food items that were found to be associated in an earlier study that investigated diet in association with resistance to penicillins and cephalosporins (amoxicillin, amoxicillin-clavulanic acid, first-generation cephalosporins and cefotaxim) in urinary tract infections caused by *E. coli*.[13]

Microbiome analysis

The microbiota was determined from the same fecal extract as described in section 2.2. Bacterial 16S rRNA variable regions V3 and V4 were amplified and sequenced using the Illumina MiSeq 2x300 base pairs protocol.[26] Phylogenetic multi-sample profiling was performed using an in-house-developed pipeline based on the QIIME 1.9.0 and USEARCH version 8.1 software packages.[27, 28] After rarefaction at 10,000 reads per sample, taxonomy was assigned using the naïve Bayesian RDP classifier[29] and the SILVA database (v119).[30] The Operational Taxonomic Unit (OTU) file was cleaned by filtering out low abundance OTUs (< 0.005% of total reads per OTUs and OTUs present in <1% of the samples). Samples with unknown information of time in the mail and samples that arrived after 3 days were excluded. More detailed information on the pipeline and characterization of the microbiota in the population is reported elsewhere.[31] The richness and Shannon alpha-diversity (diversity within a sample) was calculated for each participant and categorized in quartiles, with the lowest quartile as a reference group. Bray-Curtis beta-diversity (diversity between samples) was also determined.

Statistical analysis

Regression models

The percentage of carriership was calculated for all genes. Four different multivariable binary logistic regression association models were performed for TEM, SHV, CTX-M and CMY with possible outcome of negative (0) or positive (1) for the specific gene. All above-mentioned characteristics (number of prescriptions of the different antimicrobial drugs, age, sex, BMI, diabetes mellitus, use of PPIs, visiting abroad, use of probiotics, and the plate number on which the sample was processed) were entered in the regression models. There were some missing values for BMI, SES, use of probiotics, and visiting abroad, which were imputed using multiple imputation (n=5). The Hosmer Lemeshow test was performed to investigate goodness of fit. Regression models were performed using IBM SPSS Statistics 24.

Analysis of dietary variables

Information on diet was available for only a subset (n = 1224) of the participants, therefore, separate multivariable binary logistic regression models were created for diet that adjusted for

all variables in the above-mentioned models and for the Dutch Healthy Diet index (DHD index). This variable is a measure for the overall healthy diet pattern assessed by adherence to the Dutch Guidelines for a Healthy Diet.[32]

Analysis of microbiota data

Separate multivariable binary logistic regression models were also performed to investigate the association between the microbiota and the carriership of resistance genes (richness and alpha-diversity) corrected for the above-mentioned variables in a subset of participants (n = 1100).

Also used was the MiRKAT package, which is available in the statistical program R and tests for associations between microbiota composition and an outcome, using a semi-parametric kernel machine regression.[33] Differences in composition of the fecal microbiota were investigated (100,000 permutations) using the Bray-Curtis beta-diversity distance of participants with and without the particular BL-gene. P-values <0.05 were considered to be statistically significant.

Results

Sixty-nine percent of the Individuals of the Rotterdam Study who were asked to collect a feces sample complied with the request. The study population comprised of 1516 individuals with a median age of 63 years, of whom 886 (58.4%) were women. The participants were from all socioeconomic population groups. Of the four BLs, the most prevalent was TEM, in 804 (53.0%) of the participants. SHV was the second most common gene in 279 individuals (18.4%). The least common genes were CTX-M in 82 (5.4%) and CMY in 55 (3.6%) of the participants (distribution of Cq-values in **Figure 1)**. It was not possible to distinguish between chromosomal and plasmid-encoded CMY, thus it was not known whether these participants were carriers of a plasmid-encoded CMY or a chromosomal-encoded CMY. **Table 1** shows carriership of combinations of the different genes. Interestingly, 563 (37.1%) of the individuals did not carry any resistance gene, although amongst them could be carriers of TEM with a high Cq-value, which could not be distinguished because of the contamination of the master mix.

Associations with use of antimicrobial drugs

Use of penicillins with extended spectrum (J01CA) was associated with TEM carriership (OR 1.11; 95%CI 1.00–1.22) and use of beta-lactamase resistant penicillins (J01CF) was associated with CMY carriership (OR 1.82; 95%CI 1.08 –3.01). Furthermore, use of macrolides and lincosamides (J01F) was associated with TEM carriership (OR 1.12; 95%CI 1.00–1.25) and with SHV carriership (OR 1.24; 95%CI 1.09–1.43) and use of tetracyclines (J01AA) was associated with CTX-M carriership (OR 1.37; 95%CI 1.09 – 1.67) (**Table 2**).

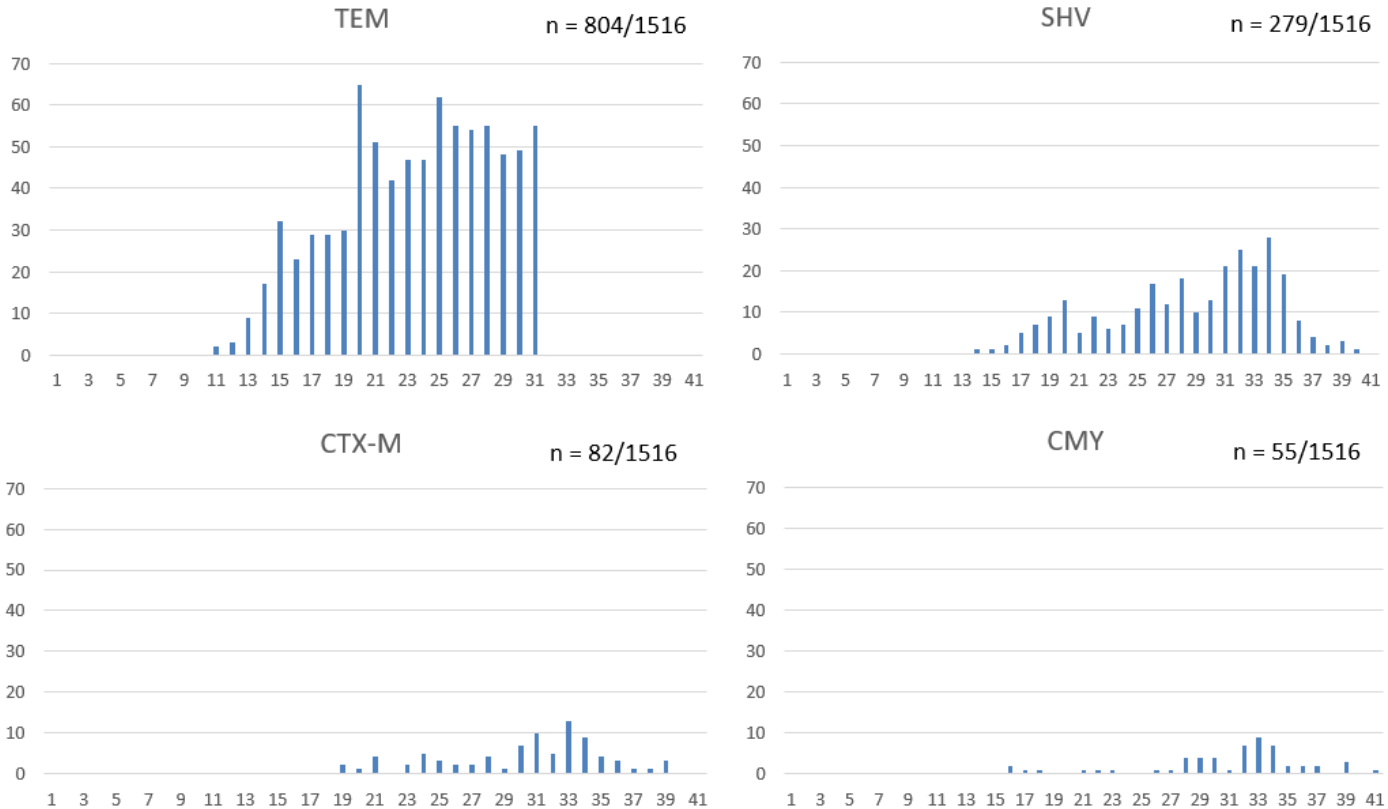


Figure 1: Distribution of the Cq-values of TEM, SHV, CMY and CTX-M in our study cohort. For TEM, 804 individuals had a Cq-value below 31.3. For SHV, CTX-M and CMY respectively 279, 82 and 55 individuals were found to be positive.

Table 1: Combinations of fecal carriership of TEM, SHV, CMY and CTX-M

	TEM	SHV	CMY	CTX-M
TEM	-			
SHV	156 (10.3)	-		
CMY	38 (2.5)	21 (1.4)	-	
CTX-M	63 (4.2)	19 (1.3)	11 (0.7)	-

Numbers (%) of individuals who have a combination of two of the genes.

Microbiota

The association between the microbiota composition and carriership was also studied. A higher richness (OR 1.17; 95%CI 1.04–1.32), borderline higher alpha-diversity (OR 1.09; 95%CI 0.99–1.23) and a different composition (beta-diversity p<0.005) were associated with TEM carriership. Furthermore, carriers of SHV had a lower richness (OR 0.84; 95%CI 0.72–0.98) and a different composition of the microbiota (beta-diversity: p=0.005) **(Table 2).**

Other determinants

Males were more frequently carriers of TEM than females (OR 1.42; 95%CI 1.10–1.76), no associations were found between sex and the other genes were found. Interestingly, use of PPIs was associated with carriership of TEM, SHV and CMY (TEM: OR 1.34; 95%CI 1.05–1.77; SHV: OR 2.17; 95%CI 1.55 – 2.87; CMY: OR 2.26; 95%CI 1.23–4.11). Finally, there was an association between diabetes mellitus and higher SHV carriership (OR 2.20; 95%CI 1.35–3.70), use of probiotics and lower SHV carriership (OR 0.47; 95%CI 0.27–0.82) and high intake of potatoes and low CMY carriership (OR 0.71; 95%CI 0.52–0.99) (**Table 2**).

Discussion

This study investigated fecal carriage of several BL-genes in a community-dwelling population-based cohort with middle-aged and elderly individuals. The results showed the most common BL-gene was TEM (53.0%), followed by SHV (18.4%). Carriership of CTX-M, which hydrolyzes third generation cephalosporins (5.4%), was far less common and carriership of CMY, which belongs to the AmpC BLs, was the least common (3.6%).

Table 2. Odd ratios for the association between several potential risk factors and fecal carriership of TEM, SHV, CMY and CTX-M

	TEM	SHV	CMY	CTX-M
Sex (ref = female)	1.42 (1.10 – 1.76)*	1.07 (0.81 – 1.50)	1.46 (0.80 – 2.70)	0.79 (0.45 – 1.26)
Age	1.01 (0.99 – 1.03)	0.99 (0.97 – 1.02)	1.02 (0.97 – 1.06)	0.99 (0.95 – 1.03)
Use of penicillins with extended spectrum (J01CA)	1.11 (1.00 – 1.22)*	0.97 (0.85 – 1.09)	0.99 (0.76 – 1.29)	1.09 (0.90 – 1.37)
Use of beta-lactamase sensitive penicillins (J01CE)	0.89 (0.69 – 1.15)	0.95 (0.70 – 1.35)	0.97 (0.51 – 1.85)	0.71 (0.39 – 1.34)
Use of beta-lactamase resistant penicillins (J01CF)	1.02 (0.78 – 1.34)	0.89 (0.62 – 1.12)	1.82 (1.08 – 3.01)*	1.04 (0.59 – 1.84)
Use of combinations of penicillins, incl. beta-lactamase inhibitors (J01CR)	1.03 (0.92 – 1.17)	0.96 (0.83 – 1.12)	1.14 (0.86 – 1.53)	1.11 (0.88 – 1.40)
Use of tetracyclines (J01AA)	1.01 (0.91 – 1.11)	0.99 (0.87 – 1.12)	1.05 (0.81 – 1.36)	1.37 (1.09 – 1.67)*
Use of sulfonamides and trimethoprim (J01E)	0.99 (0.85 – 1.13)	1.03 (0.86 – 1.22)	0.99 (0.67 – 1.46)	1.10 (0.82 – 1.48)
Use of macrolides and lincosamides (J01F)	1.12 (1.00 – 1.25)*	1.24 (1.09 – 1.43)*	0.96 (0.72 – 1.30)	1.07 (0.84 – 1.32)
Use of fluoroquinolones (J01MA)	1.08 (0.93 – 1.29)	1.00 (0.82 – 1.21)	0.60 (0.35 – 1.03)	1.26 (0.94 – 1.71)
Use of nitrofuran derivatives (J01XE)	1.13 (0.98 – 1.30)	1.10 (0.93 – 1.32)	0.97 (0.65 – 1.43)	0.74 (0.51 – 1.05)
BMI	1.01 (0.99 – 1.04)	1.00 (0.98 – 1.03)	0.97 (0.91 – 1.04)	0.96 (0.91 – 1.01)
Diabetes	1.14 (0.67 – 1.76)	2.20 (1.35 – 3.70)*	1.25 (0.41 – 3.74)	1.25 (0.48 – 3.46)
SES	1.06 (0.95– 1.20)	1.01 (0.87 – 1.17)	1.02 (0.75 – 1.37)	1.20 (0.92 – 1.54)
Use of proton pump inhibitors	1.34 (1.05 – 1.77)*	2.17 (1.55 – 2.87)*	2.26 (1.23 – 4.11)*	0.61 (0.33 – 1.16)
Use of probiotics	1.08 (0.75 – 1.51)	0.47 (0.27 – 0.82)*	1.72 (0.78 – 3.80)	0.77 (0.34 – 1.74)
Abroad				
Within Europe	1.01 (0.75 – 1.41)	1.16 (0.76 – 1.70)	Not possible[†]	0.75 (0.35 – 1.64)

	TEM	SHV	CMY	CTX-M
Outside Europe	1.66 (0.83 – 3.13)	1.89 (0.95 – 4.00)	Not possible†	1.64 (0.56 – 5.08)
Microbiota ‡				
Richness	1.17 (1.04 – 1.32)*	0.84 (0.72 – 0.98)*	0.87 (0.56 – 1.37)	1.05 (0.83 – 1.32)
Alpha-diversity	1.09 (0.99 – 1.23)	0.90 (0.77 – 1.05)	1.02 (0.76 – 1.36)	0.97 (0.76 – 1.22)
Beta-diversity	P < 0.005*	P = 0.005*	P > 0.05	P > 0.05
Food item¥				
Chicken	0.95 (0.84 – 1.07)	1.03 (0.88 – 1.20)	0.96 (0.70 – 1.33)	0.80 (0.61 – 1.05)
Cheese	1.00 (0.80 – 1.12)	0.92 (0.79 – 1.08)	0.83 (0.60 – 1.15)	0.89 (0.68 – 1.17)
Potatoes	0.93 (0.82 – 1.05)	0.92 (0.79 – 1.09)	0.71 (0.52 – 0.99)*	1.07 (0.80 – 1.44)
Vegetables	0.89 (0.78 – 1.03)	1.03 (0.85 – 1.21)	1.23 (0.86 – 1.77)	1.06 (0.81 – 1.38)

This table shows the multivariable model for associations with carriership of the resistance genes TEM, SHV, CMY and CTX-M. Age is the age at moment of sampling. Prescriptions of antimicrobial drugs before sampling were categorized as no use, 1 prescription, 2 prescriptions and 3 or more prescriptions. Because of the low number of prescriptions, the prescriptions of beta-lactam sensitive and beta-lactam resistant penicillins were categorized into: no prescriptions, 1 prescription, 2 or more prescriptions. Diabetes mellitus was scored as the use of hypoglycemic drugs. Use of proton pump inhibitors is scored as a prescription of a proton pump inhibitor in the 90 days before sampling or use according to the questionnaire. Use of probiotics is scored as use of any probiotic in the three days before sampling according to a questionnaire. Abroad within or outside Europe was scored as travelling abroad in the month before sampling according to the questionnaire. The model was further adjusted for plate number of the sample in the analysis. The Hosmer-Lemeshow test showed a good fit.

* Statistically significant (p < 0.05); ‡ The analysis of the microbiota variables was performed in a two separate models (for richness and alpha-diversity, both in quartiles), since these data was only known in a subset of participants (n=1100). The multivariable model was adjusted for all variables that were used in the main analysis. ¥ The analysis of the trends of the food items was also performed in a separate model asthese data were also only known in a subset of participants (n = 1224). The shown variables were adjusted for all variables that were used in the main analysis. † It was not possible to add this variable in this analysis because of the low number of cases.

Carriership varies greatly worldwide, even in a small country like The Netherlands, varied results have been reported. Notably, most studies were not based on PCR, but on culturing of specimens. TEM carriership in the current population was 53.0%, and SHV carriership was 18.4%. TEM carriership may have been slightly higher in this study as it was not possible to distinguish individuals with high Cq-values for TEM due to the contaminated master mix. TEM and SHV families consist of both small-spectrum beta-lactamases and ESBLs [4] and the method of analysis could not distinguish between the two. TEM-1 and TEM-2 are the most common TEM-genes and CTX-M is known to be the most common ESBL in the last decades. Therefore, most of the positive

TEM PCRs demonstrate are likely to indicate the presence of TEM-1 or TEM-2, which are small-spectrum BLs.[34-37] Hyperproduction of TEM-1, which could not be demonstrated nor excluded in this study has been reported as a common resistance mechanism to amoxicillin-clavulanic acid.[38] The percentage of carriership for TEM was also in agreement with resistance to ampicillin/amoxicillin, which was shown to vary between 40-50% in clinical cultures, depending on the population, microorganism and tissue, in The Netherlands in 2019.[39]

CTX-M and CMY carriership was far less common in the current study population (5.4% and 3.6%, respectively). These are extended-spectrum and AmpC BLs and can be more easily compared with other populations. These rates are in line with the most recent national data on ESBL-producing Enterobacterales, showing rates of approximately 3% in GP patients and approximately 5% in hospital patients (excluding ICU-patients).[39] Furthermore in a study of healthy volunteers from The Netherlands, 9.5% were carriers of isolates with ESBL-genes, which were predominantly CTX-M genes and only 1.1% was CMY.[16] In another Dutch study, 16.3% of patients screened at hospital admission were carriers of ESBL genes. The most recent Dutch study showed that nearly 3% of the *E.coli* isolates from cultures in outpatients clinics produced ESBLs versus over approximately 5.5% of the cultures from ICUs. These percentages were over 4% and approximately 9% for *Klebsiella* isolates.[40]

Carriership of ESBL-genes, however, varies strongly between regions. A Spanish study with in- and outpatients showed that 5.1% of the individuals carried ESBL-genes, of which CTX-M genes were the most prevalent (96.2%).[17] In healthy adults in Japan, 6.4% were carriers of ESBL-genes, of which 92.6% were CTX-M[18] and in an German community, there was 6.3% ESBL-carriership, of which 95.2% were CTX-M.[19] Furthermore, CTX-M carriership was 33.9% in outpatients in Turkey,[41] 50.5% in healthy individuals in China,[42] and 52.1% and 65.7% in two Thai populations.[43, 44] In the Turkish outpatients and in one of the Thai communities, AmpC carriership was substantially lower than ESBL-carriership (Turkish outpatients 1.9%, Thai community 6.2%).[41, 44] The lower prevalence of ESBL in the Netherlands is most likely due to the presence of national antimicrobial stewardship programs and is in line with the low overall antibiotic use and general antimicrobial drug resistance rates.

In the current study, fecal samples (rather than clinical cultures) from healthy individuals were investigated using qPCR and the complete reservoir of genes present in the gut was studied. Although culturing is still often seen as a golden standard, qPCR is less laborious and thus more feasible to use in a large population with healthy individuals. Using qPCR rather than clinical culture in the current study might explain why CMY is more common in our study in proportion with CTX-M than in all above mentioned studies, indicating that carriership of CMY might be more frequently present than is assumed from studies investigating clinical cultures. However, a limitation might be that CMY is homologous to the chromosomally encoded AmpC gene of *Citrobacter freundii* and therefore also carriers of *C. freundii* might have scored positive by our

PCR. Also, the current method could further be limited because the primers could possibly not detect all members of the CMY-family; in which case the percentage of carriers would be underestimated. However, an increase in AmpC-producers was also seen in clinical cultures from Canadian hospitals, both in absolute numbers and relative to ESBLs.[45] An 86% sensitivity and 98% specificity was found when comparing culturing with qPCR in Dutch ICU patients with 5% carriership, although rectal swabs rather than fecal samples were used.[46]

Unexpectedly, the results of the current study indicated few associations between the use of beta-lactam antibiotics and the carriership of these genes, except for use of penicillins with extended spectrum, such as amoxicillin and TEM carriership. However, as the study comprised a healthy population, antimicrobial drugs were used most of the time in the distant past. Carriership of ESBL-genes has been shown to be easily be acquired, but also lost again, resulting in a relatively short duration of carriership with the duration dependent on the type of resistance gene.[47]

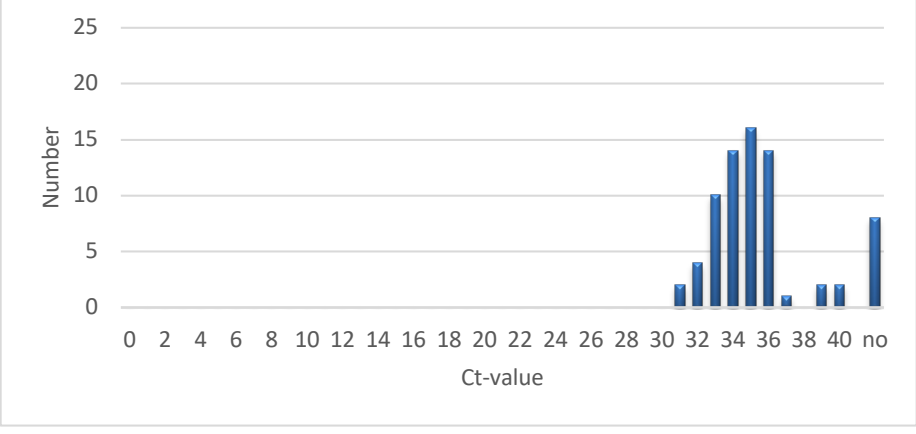
Associations were found between tetracyclines use and CTX-M and between macrolides and lincosamides use and a higher prevalence of the TEM and the SHV gene. There was also an association between the use of probiotics and lower carriership of SHV. Although, these associations do not seem to be very obvious, a study with a CTX-M-15-producing *E.coli* in a mouse intestinal colonization model has shown that clindamycin can promote the overgrowth of this *E.coli*, probably because its strong impact on the anaerobic flora.[48] Furthermore, the group of macrolides and lincosamides have been shown to have a profound effect on the human gut microbiota.[49] The microbiota has been suggested to play a role in the composition of the resistome, and dysbiosis has been hypothesized to play a role in horizontal gene transfer.[8, 50] There were also associations between the microbiota composition and TEM and SHV carriership, possibly because of the higher power for this groups in the analysis, but unexpectedly, TEM carriership was found at a higher diversity.

The study also showed associations between the use of PPIs and carriership of TEM, SHV and CMY. PPIs have been shown to play a role in carriership of ESBL-genes and are also known to have an effect on the microbiota, decreasing the diversity.[51, 52] An association with a high intake of potatoes and lower carriership of CMY was found in our study. This also points into the direction of the microbiota as a diet rich in vegetables, fibers and polysaccharides have been proposed as beneficial for the gut microbiota.[53-56] Thus, potential associations were found between factors that might affect the gut microbiota and carriership of one, but not all, of the genes. Further associations between diet and antimicrobial resistance in clinical cultures were found in an earlier study.[13] However, as qPCR was used in this study, it is not known in which bacteria these genes reside and whether these bacteria are clinically relevant. Furthermore, because of the skewed distribution of the gene load, the presence or absence of the genes was denoted as yes/no, thereby ignoring the substantial differences in Cq-values between

individuals. This could have led to dilution of effect-estimates. In addition, the cut-off for TEM was set at 31.3 and the samples with a higher Cq-value were considered negative. In conclusion, the microbiota might play a role in the carriership of resistance genes, which could result in infections with resistant bacteria, but this role is complex and must be elucidated further.

In this otherwise healthy community-dwelling population TEM was the most prevalent BL-gene in the resistome, followed by SHV, whereas the prevalence of fecal carriership of the ESBL, CTX-M and the AmpC BL, CMY was lower, but in line with resistance in clinical cultures according to national data. Fecal carriership of BL-genes was higher with concurrent use of PPIs. Additional studies should be conducted to further investigate the role of the gut microbiota in the composition of the resistome, because this could be an important target for lowering antimicrobial resistance. Unfortunately, it was not possible to distinguish how TEM and SHV contribute to ESBL-carriership. Furthermore, carriership of carbapenemase genes, such as VIM, IMP, KPC and OXA-48, is an interesting topic for future research, but as carbapenem resistance in clinical cultures is very low in The Netherlands,[37] this was not investigated in the current study.

Supplementary figure 1: Distribution of Ct-values for TEM of the blanc reactions



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Chapter 4.4

Composition of the Gut Microbiota as a Risk Factor for Urinary Tract Infections in Women

Marlies Mulder, Djawad Radjabzadeh, Robert Kraaij, Joyce van Meurs, Annelies Verbon, Bruno Stricker

In preparation

Abstract

Objective Urinary tract infections (UTIs) are very common infections, mostly affecting women. It has been suggested that the gut microbiota plays a role in the development of UTIs, but the relationship is unclear. Here, we investigated whether the composition of the gut microbiota is associated with the development of UTIs.

Methods We obtained a fecal sample (start date of the study) and pharmacy data from 819 women of the Rotterdam Study, a population-based cohort study in 14,926 middle-aged and elderly individuals. We used Cox regression models to investigate whether the composition of the gut microbiota was associated with time to occurrence of the first UTI (defined as a prescription for nitrofurantoin, trimethoprim, fosfomycin, ciprofloxacin, amoxicillin-clavulanic acid or sulfamethoxazole-trimethoprim) in women without a recent UTI with fecal sampling as starting point of the follow-up.

Results In our population, 423 out of 819 women had not been treated for a UTI in the 4-year period before fecal sampling. 120 of these 423 women (28.4%) had one (or more) UTIs during the follow-up time. The composition of the microbiota (beta-diversity) was significantly associated with occurrence of a first UTI during follow-up. The alpha-diversity was significantly associated when adjusted for technical covariates (HR 0.85; 95%CI 0.77 – 0.95), but not in the complete model (HR 0.91; 95%CI 0.81 – 1.02), probably because the association was confounded by previous use of non-UTI antimicrobial drugs HR 1.42; 95%CI 1.23 – 1.65 per time category). Use of proton pump inhibitors was also associated with occurrence of UTI (HR 1.65; 95%CI 1.00 – 2.69).

Conclusions Our data suggest that the composition of the gut microbiota plays a role in the occurrence of a first UTI in women without a recent UTI history. For future research the gut microbiota may be a target for preventive interventions.

Introduction

Urinary tract infections (UTIs) are very common infections in humans, especially in women, resulting in a considerable burden of disease. Indeed, in Dutch women, UTIs were the most common reason to consult a general practitioner (GP) [1] and they are an important reason for general practitioners (GPs) to prescribe antimicrobial drugs [2, 3]. UTIs in women are frequently occurring in all age groups, with peaks in young adulthood and increasing with age after menopause.[4] Furthermore, a substantial group of women suffer from recurrent UTIs. For example, in a study with female college students, who suffered from their first UTI, 27% had a first recurrence within 6 months.[5]

Several host risk factors for UTIs have been reported, such as sexual activity, urinary tract abnormalities, and having a mother with recurrent UTIs.[6, 7] Next to host factors, the virulence of the uropathogens plays a role. For example, *E.coli* strains that cause UTIs have specific virulence factors such as specific adhesins, toxins and polysaccharide coatings. Furthermore, some studies suggest that these *E.coli* are able to escape from the defense of the innate immune system.[8, 9]

It has been hypothesized that the vaginal microbiota plays a role in UTIs especially in post-menopausal women. Vaginal estrogens are suggested to reduce the risk for UTIs as is the use of probiotics with *Lactobacillus* species to restore the normal vaginal flora. It has also been hypothesized that uropathogens have their origin from the gut microbiota but the exact relationship is unclear. The administration of probiotics (including different *Lactobacillus* species) was investigated for prevention of UTIs with varying results. One advantage was that risk of selection of resistant bacteria was lower in women using probiotics than in women using antimicrobial drugs prophylaxis. [10-12] Furthermore, the microbiota in the human gut is composed of numerous different bacteria that play a role in health and disease.[13] A recent study in kidney transplant patients showed that a 1% relative gut abundance of *E.coli* is a risk factor for *E.coli* bacteriuria and UTIs.[14] Also, a study in 106 children has shown differences in gut microbiota between children with febrile UTI and healthy children.[15]

Although it has been suggested that the gut microbiota plays a role in the development of UTIs, its relationship is unclear. Here, we investigated whether the composition of the gut microbiota is associated with the development of UTIs in women without a recent history of UTIs.

Materials and methods

Study population

The fecal samples in this study were obtained from participants of the third cohort (RSIII) of the Rotterdam Study, a prospective population-based cohort study including 14,926 individuals of 45 years and older from the Ommoord area in Rotterdam, The Netherlands. In this study, we

included women from the third cohort (n= 2252 women), who were asked to send in a fecal sample. The Rotterdam Study is extensively described elsewhere.[16]

Gut microbiota composition

The participants collected one fecal sample at home using a Commode Specimen Collection System (Covidien, Mansfield, MA) in the period May 2021 – August 2014 and sent it through regular mail to the Erasmus MC. *After receipt, the samples were stored at -20°C. Approximately 300mg of feces was homogenized in stool stabilizing buffer.* The Arrow DNA kit (Arrow DNA; DiaSorin S.p.A., Saluggia, Italy) was used to perform automated DNA-isolation according to the manufacturer's instructions. This included bead-beating in Lysing Matrix B tubes (with 0.1mm silica beads) (MP Biomedicals, LLC, Bio Connect Life Sciences, Huissen, The Netherlands). The hypervariable regions V3 and V4 of the (bacterial) 16S rRNA gene were amplified and sequenced using the Illumina MiSeq 2x300 base pairs protocol (FADROSH, PMID: 24558975).

Raw reads from Illumina MiSeq were demultiplexed using a custom script to separate sample fastq files based on the dual index. Primers, barcodes and heterogeneity spacers were trimmed off using tagcleaner v0.16.[17] Trimmed fastq files were loaded into R v4.0.0 with the DADA2 package.[18] Quality filtering was performed in DADA2 using the following criteria: trim=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE. Filtered reads were run through the DADA2 Amplicon Sequence Variant (ASV) assignment tool to denoise, cluster and merge the reads. ASVs were assigned a taxonomy from the SILVA version 138.1 rRNA database using the RDP naïve Bayesian classifier.[19, 20] The resulting data tables were combined into a phyloseq object using Phyloseq.[21] Samples that were >=8 days in the mail, samples without information on date of sampling, samples with less than 4.5K reads or samples, or samples that lost more than 50% of reads in the pipeline were excluded. More information on the microbiota analysis van be found here.[22] The Shannon alpha-diversity (categorized in sextiles) and Bray-Curtis beta-diversity were calculated.

Prescriptions information

Prescription *data were obtained from all community pharmacies in the Ommoord area, consisting of all out-patient prescriptions since 1st January, 1995 until 8 February 2021.* Antimicrobial drug use was categorized *as antimicrobial drugs that are prescribed for UTIs (*nitrofurantoin, fosfomycin, trimethoprim, ciprofloxacin, amoxicillin-clavulanic acid and sulfamethoxazole-trimethoprim), which are all options for the treatment of UTIs according to the Dutch national guidelines for GPs, [23] and non-UTI antimicrobial drugs that are not (all antimicrobial drugs that are not prescribed for UTIs).

Cohort definition

The study cohort consisted of all women of whom we had a fecal sample and who had not filled a prescription for an antimicrobial drug that was likely to be prescribed for a UTI in the 4 years before sampling. Cases were women who received a first prescription for an antimicrobial drug

for a UTI in the study period after fecal sampling. The remainder of the cohort consisted of women who did not receive such an antimicrobial drug during follow-up. The time between fecal sampling and this first prescription for a UTI in cases was calculated and defined as time-to-event. Non-cases were censored on 1 January 2021, or date of death, whichever occurred first. (**Figure 1**). Both cases and non-cases were allowed to have used other antimicrobial drugs in the 4 years preceding fecal sampling.

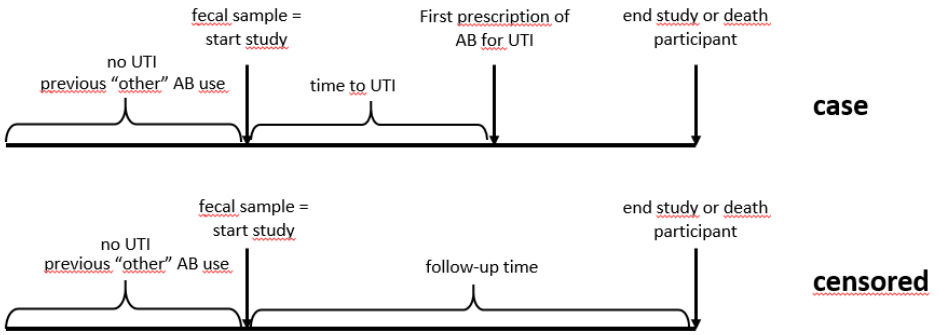

Figure 1: Study design. Women from the Rotterdam study were included when a fecal sample was available (start study) and when no antimicrobial drug for a UTI was prescribed in the 4 years before. The time to first UTI or time to censoring (end study or death participant) was calculated. The analyses were adjusted for other potential risk factors: age, BMI, diabetes, use of proton pump inhibitors (at moment of sampling) and previous use of "other" antimicrobial drugs (other than for UTIs), which was categorized into 0-12 months, 12-24 months, 24-48 months > 48 months or no use.

Confounders

*A*ge and BMI were obtained from the visit of our study center at the time of fecal sampling. The use of other (non-UTI) antimicrobial drugs in 4 years before fecal sample was categorized into no use; use *0-1; 1-2; 2-4; and >4 years before sampling. Furthermore, we obtained data on glucose-lowering drugs, to determine which patients have diabetes, because diabetics are known to be more prone to UTIs. We also obtained data on use of proton pump inhibitors (PPIs) because these are known to have an important effect on the gut microbiota.*

Statistical analysis

A Cox regression analysis was performed to investigate whether the (categorized) alpha-diversity of the fecal microbiota was associated with the occurrence of a UTI during follow-up. Other potential risk factors that were added to the model were age, BMI, diabetes, use of other (non-UTI) antimicrobial drugs before fecal sampling (categorized), and use of PPIs. Furthermore, several technical confounders (time in mail, batch number, number of reads) were added in the

model. We also performed *a semi-parametric kernel machine regression, using* the MiRKATS package, *that tests for associations between the beta-diversity and survival outcomes in the same model as described for the alpha-diversity.[24] A few samples with missing information on BMI (1) and time in the mail (13) were imputed with the median value of this variable.*

Because amoxicillin-clavulanic acid could also be prescribed for another indication than a UTI, we performed a sensitivity analysis, in which this antimicrobial drug was not used in the case definition. Thus, in this analysis individuals using only amoxicillin-clavulanic acid as antibiotic treatment after fecal sampling were considered non-cases, and for individuals who had a prescription of another antimicrobial drug than amoxicillin-clavulanic acid for a UTI the time-to-event was adjusted to this date.

P-values < 0.05 were considered to be statistically significant.

Results

Fecal samples of 1410 individuals were obtained; 819 were women with a median age of 62.6 (IQR 58.3 – 66.1) years old. Of these 819 women, 423 (51.6%) were included in this study, since they were not prescribed any antimicrobial drugs for a UTI in the 4 years before fecal sampling. The median age of this group was 62.6 (IQR 58.4 – 66.2) years. In this group, 120 women (28.4%) were prescribed antimicrobial drugs for a UTI after a median of 32.6 (IQR 13.9 – 63.4) months during follow-up. The most frequently filled antimicrobial drug against UTIs during follow-up was nitrofurantoin (58.3%). Censoring because of death of the participant or because of end of study was after a median time of 90.7 (83.5 – 95.5) months.

Use of antimicrobial drugs which were prescribed for other indications than for UTIs was common; 62.4% of all women were prescribed at least one (non-UTI) antimicrobial drug before start of the study (fecal sampling). Furthermore, few women had diabetes mellitus (10; 2.4%) and 45 (10.6%) used PPIs (**Table 1**).

In women without recent UTIs, a higher alpha diversity was associated with a lower hazard of occurrence of the first UTI (HR 0.85; 95%CI 0.77 – 0.95), when only adjusted for the technical covariates time in the mail, batch, and number of reads. However, this was not significant in the complete model (HR 0.91; 95%CI 0.81 – 1.02). The strongest confounder for this association was previous use of non-UTI antimicrobial drugs (HR 1.42; 95%CI 1.23 – 1.65 per time category). Also use of PPIs was associated with the occurrence of first UTI (HR 1.65; 95%CI 1.00 – 2.69) Finally, the composition (beta-diversity) of the microbiota was significantly associated with occurrence of the first UTI in the complete model. No association was found for age, BMI and diabetes (**Table 2**).

Table 1: Characteristics of the study population

Variable	All (n = 423)	Cases (n = 120)	Censored (n = 303)
Age (years), median (IQR)	62.6 (58.4 – 66.2)	63.1 (57.5 – 66.5)	62.5 (58.8 – 66.2)
Women with UTI during follow-up, n (%)	120 (28.4)		
Time to UTI (months), median (IQR)	-	32.6 (13.9 – 63.4)	-
First antimicrobial drug for UTI in follow-up:			
none	303 (71.6)	0 (0)	303 (100)
Nitrofurantoin, n (%)	70 (17.4)	70 (58.3)	-
Trimethoprim, n (%)	2 (0.5)	2 (1.7)	-
Fosfomycin, n (%)	9 (2.1)	9 (7.5)	-
Ciprofloxacin, n (%)	6 (1.5)	6 (5.0)	-
Amoxicillin-clavulanic acid, n (%)	32 (7.6)	32 (26.7)	-
Trimethoprim-sulfamethoxazole, n (%)	1 (0.2	1 (0.8)	-
Time to censoring (months), median (IQR)	-	-	90.7 (83.5 – 95.5)
BMI, median (IQR)	26.5 (23.9 – 29.2)	26.4 (24.2 – 29.3)	26.6 (23.8 – 29.2)
Diabetes, n (%)	10 (2.4)	3 (2.5)	7 (2.3)
Use of other antimicrobial drugs before fecal sample, n (%)	264 (62.4)	86 (71.6)	178 (58.7)
Use of proton pump inhibitors, n (%)	45 (10.6)	22 (18.3)	23 (7.6)

Table 2: HRs for the association between several potential risk factors and first occurrence of a UTI.

Variable	Model 1	Model 2
Alpha-diversity	0.85 (0.77 – 0.95)*	0.91 (0.81 – 1.02)
Age	1.01 (0.98 – 1.05)	1.01 (0.98 – 1.05)
BMI	1.00 (0.97 – 1.04)	0.98 (0.95 – 1.02)
Diabetes	1.22 (0.39 – 3.87)	1.46 (0.46 – 4.68)
Proton pump inhibitors	2.30 (1.44 – 3.66)*	1.65 (1.00 – 2.69)*
Previous use of other antimicrobial drugs	1.50 (1.31 – 1.73)*	1.42 (1.23 – 1.65)*
Beta-diversity	p <0.005*	p = 0.02*

Table 2 shows the results of the Cox regression for time to first UTI, resulting in hazard ratios for the alpha-diversity (according to Shannon), age, BMI, diabetes, proton pump inhibitors and antimicrobial drug use before sampling. In a separate analysis, the association of the beta-diversity (Bray-Curtis) with time to UTI was analyzed adjusted for age, BMI, proton pump inhibitor, number of other drugs and antimicrobial drug use before sampling, resulting in a p-value. Note that diabetes was left out as confounder in this analysis, since the number of diabetics was too low to obtain a result. Cases were considered to be women who were prescribed nitrofurantoin, fosfomycin, trimethoprim, ciprofloxacin, amoxicillin-clavulanic acid and sulfamethoxazole-trimethoprim. * significant $p < 0.05$.

We also performed a sensitivity analysis, in which amoxicillin-clavulanic acid was not included as a proxy indicator in the case definition of having a UTI, because it can also be prescribed for other indications. This analysis resulted in comparable estimates. However, the use of PPIs was no longer statistically significantly associated with the occurrence of the first UTI, whereas the composition of the beta-diversity was only borderline significant in the complete model (data not shown).

Discussion

In this study, we found that the composition of the microbiota (beta-diversity) was significantly associated with the occurrence of the first UTI after fecal sampling. Another risk factor was the use of other antimicrobial drugs (that were prescribed for another indication than for UTIs) in the period before fecal sampling. The alpha-diversity was significantly associated with the occurrence of UTIs when only adjusted for the technical covariates, but not in the complete model when adjusted for, amongst others, previous use of non-UTI antimicrobial drugs and use of PPIs.

In the last decade, the interest in the role of the gut microbiota as a risk factor for UTIs has increased. In addition to studies in patients after transplantation and in children described in the introduction section,[14] [15] we here show an association between the composition of the gut microbiota and the occurrence of a first UTI in middle-aged and elderly community-based women without a UTI in the preceding 4 years. Other more indirect studies also underline this hypothesis, for example, the use of cranberries was shown to modulate the gut microbiota by reducing the abundance of Enterobacteriales and increasing the abundance of Bacteroidaceae.[25] Cranberries have been shown to reduce the risk of UTIs.[26] Furthermore, it was shown that fecal transplantation to treat recurrent *Clostridium difficile* infections resulted in decreased recurrent UTI frequency.[27] There is also a possible interplay between the urinary and the vaginal microbiota, which is known to affect a women's susceptibility to UTIs. Earlier, both the vaginal and urinary tract microbiota were suggested to be a potential reservoir of uropathogenic *E.coli* and to play a role in the protection against UTIs.[28, 29] However, a study that investigated the gut microbiota of 40 postmenopausal women with recurrent UTIs found that the gut microbiota was not predictive for new UTIs, when comparing women with 4 or more infections with women without UTI during follow-up. [30] Although, the number of patients in this study was low, it made us speculate that the gut microbiota is playing a less important role in women with recurrent UTIs than in women who suffer from a first UTI after a disease-free period. Indeed, we found an association between beta-diversity of the gut microbiota and the occurrence of UTIs in women with a UTI free period of at least 4 years.

We also found an association between a lower alpha-diversity and the occurrence of a UTI when only adjusted for the technical covariates, but not in the complete model. This association was strongly confounded by recent use of antimicrobial drugs for other indications than UTIs. This is interesting since the use of non-UTI antimicrobial drugs, and especially the group of macrolides and lincosamides have been shown to have long-term effects on the gut microbiota.[31] Also, the use of PPIs, which was also significantly associated with the occurrence of a first UTI has been associated with effects on the gut microbiota.[32] Therefore, it is possible that the gut microbiota is an intermediate step between the association of use of non-UTI antimicrobial drugs and PPIs, and UTIs. Alternatively, the use of antimicrobial drugs and PPIs may be an indication that the overall health of the individual is less than that in individuals who do not use such drugs.

A strength of this study is that we investigated a large and prospective population-based cohort with filled prescription data from pharmacies. A limitation might be that we used the prescription data as a proxy indicator of UTIs. However, the antimicrobial drugs that we studied are mostly exclusively prescribed for UTIs. The only exception might be amoxicillin-clavulanic acid, which is in some cases prescribed by GPs for other infections. However, a sensitivity analysis with cases without amoxicillin clavulanic acid use showed similar results, except that beta-diversity was only borderline significant, which could be explained by the lower power of this analysis. However, it is possible that we missed prescriptions from individual elderly who moved to nursery homes. This would mean that censored individuals could possibly be undiscovered cases, which would only dilute our estimate and thus strengthen our results.

In conclusion, we found that both the composition of the gut microbiota and use of non-UTI antimicrobial drugs and PPIs, are associated with the occurrence of an UTI in women without a recent UTI history. Given the influence of both antibiotic drugs and PPIs on the composition of the gut microbiota, our data suggests that the gut microbiota play a role in the pathogenesis of a first UTI. Further studies are needed to elucidate the complex interplay of the microbiota in the occurrence of (recurrent) UTIs in women.

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Chapter 5

General Discussion

Urinary tract infections (UTIs) are among the most common infections worldwide. Apart from a peak in the incidence in young women, incidence increases with age, especially after menopause. A simple cystitis might seem to be a rather innocent infection which is easy to treat, but it can evolve into pyelonephritis or even into a life-threatening urosepsis.[1] The incidence of UTIs in general practice in The Netherlands in 2018 was 20 per 1000 individuals for men and 125 per 1000 for women.[2] Infections of the lower respiratory tract and UTIs are the most common reason for individuals of 65 years and older with fever to present themselves at the emergency department.[3] In the US, approximately 0.9% of hospital admissions is due to UTIs and interestingly the number of admissions have increased over time.[4] Furthermore, studies in Israel and Europe have shown that in 12% and 14% of all individuals who were admitted to the ICU with sepsis, the urinary tract was the primary site of infection.[5, 6] Thus, on a population level, UTIs account for a substantial burden of morbidity, mortality and economic costs.[7] Besides, UTIs also have a negative impact on individual quality of life. Also, recurrent UTIs are associated with symptoms of anxiety and depression.[8] Unfortunately, due to the increase in antimicrobial resistance (AMR) rates, treating UTIs will become increasingly difficult. In fact, the increase of hospital admissions in the US due to UTIs was suggested to be caused by the fact that more individuals needed intravenously administered antimicrobial drugs due to higher resistance rates.[4]

To stop a further increase or, even better, to diminish AMR, it is critical to investigate which factors promote and which factors can prevent the development of AMR. The focus of this thesis was therefore on investigating factors that can possibly decrease AMR in UTIs. First of all, it is known that antimicrobial drug use itself is a risk factor for AMR.[9] Therefore our first target was to investigate the prescribing of antimicrobial drugs for UTIs. Second, we investigated the role of antimicrobial drugs and other factors in the development of AMR in UTIs. Our third target was to study the gut and the urinary microbiota, which might play a role in the keeping of (resistant) urinary pathogens and resistance genes. We also studied the potential role of the microbiota in the development of UTIs, since it is important to understand why some women suffer from UTIs more often than others. Knowledge on how to prevent these might result in a reduced need for antimicrobial drug use and consequently, a smaller chance of selection of resistant pathogens.

Target 1: Antimicrobial drug use in urinary tract infections

One of the most important strategies in lowering AMR is good antimicrobial stewardship, which includes the correct choice of antimicrobial drug and the correct duration of use. In chapter 2, we used the large Dutch computerized primary care database IPCI to study the prescribing of antimicrobial drugs for UTIs by GPs. Interestingly, we found that prescribing was in line with the recommendations and also in line with changes in recommendations in the Dutch national guideline, especially in most recent years. A study in another Dutch database in 2001 had already

shown that approximately 75% of the prescriptions for cystitis were first-choice drugs according to the Dutch guideline.[10] These results suggest that Dutch GPs know that it is important to follow the guideline, most likely the result of a strong effort in antimicrobial stewardship programs in the Netherlands. However, this is not true for all countries. For example, a 3-fold difference was shown to be present in European countries with the lowest and highest consumption of antimicrobial drugs.[11] Furthermore, a study in France among GPs showed a large intervariability in the prescribing of antimicrobial drugs,[12] a factor that we could unfortunately not investigate in our study. Another group of infections, in which antimicrobial stewardship is widely studied are the respiratory infections, which are even more common than UTIs. Misuse of antimicrobial drugs has been reported in several studies, which estimated unnecessary prescribing in 50-90% of prescriptions.[11] These findings show the large need for the implementation of antibiotic stewardship programs for UTIs and other infections worldwide.

In chapter 2, we also found an apparent increase in total prescriptions for UTIs over the years. This might have been caused by an improvement in coding in more recent years, but the trend was still present when we controlled our analysis for this potential bias. This trend has also been shown by two other Dutch GP database studies,[13, 14] and additionally the number of contacts with a GP for UTIs has also increased in this period.[15] It is unclear yet what caused this increase Possibly, it is caused by the ageing population with individuals with more comorbidities. However, this can only have caused a part of the increase, since the trend was also shown in the younger age groups. Furthermore, individuals might suffer more often from recurrent infections, because complete eradication is more difficult due to an increase in resistant pathogens. It is also possible that individuals visit their GP more easily and expect to be prescribed antimicrobial drugs. Furthermore, GPs might prescribe more easily antimicrobial drugs for UTIs. However, a wait-and-see policy or 'postponed' prescription are options for otherwise healthy women with cystitis according to the Dutch guidelines.[16, 17] The most recent version of April 2020 even reviews the pros and cons of the wait-and-see policy and 'postponed' prescription. The authors conclude that although women do recover more quickly when using antimicrobial drugs and might have a slightly lower risk (risk difference of approximately 1.6%) of pyelonephritis, the avoidance of antimicrobial drug use could still be an important reason for a wait-and-see policy.[18] Also, a Dutch study has shown that 37% of otherwise healthy women with cystitis postponed treatment with antimicrobial drugs and 55% of these women had not have filled the antimicrobial drug prescription after a week.[18, 19] In the future, it will be very important to find out whether the potential increase in UTIs really occurs and if yes, to elucidate the reason behind this increase in order to develop an intervention.

Target 2: Risk factors for resistance in urinary tract infections

Antimicrobial drug use

Antimicrobial drug use can result in AMR to this particular antimicrobial drug.[9, 20] Bacteria are able to respond to many different environmental threats and it is, therefore, not surprising that when exposed to antimicrobial drugs, they are able to evolve in such a way that they can counteract the effects of these drugs. Especially, since many antimicrobial drugs originate from natural sources, meaning that antimicrobial peptides have been present in the environment of the bacteria for centuries. However, often, mutations in bacteria, including the ones that result in AMR come with a reduction in bacterial fitness, so without selective pressure the mutants are less likely to survive in a bacterial population.[21] This is, for example, suggested for fosfomycin and nitrofurantoin.[22, 23] However, when an individual is treated with antimicrobial drugs, the selective pressure causes the subset of bacteria that can resist the antimicrobial drug to survive.[24]

In this thesis, we found several associations between use of antimicrobial drugs and resistance to the drug itself. In chapter 3.1, we showed that use of 2 or 3 or more prescriptions of fluoroquinolones, such as ciprofloxacin, is associated with ciprofloxacin resistance in community-acquired UTIs. In a separate model, it was shown that mainly the prescriptions in the year before study were important in ciprofloxacin resistance. Furthermore, in chapter 3.2 we showed similar results for trimethoprim use and trimethoprim resistance, although in this case the association was for >3 prescriptions and for 1-3 months before culturing. Although, we cannot compare these results one-to-one, it has been shown that AMR to one drug is more easily acquired than antimicrobial resistance to another one due to the different AMR mechanisms.[24]

Next to the known and obvious associations between use of an antimicrobial drugs and resistance to this particular antimicrobial drug, we showed in chapter 3.2 that the use of antimicrobial drugs belonging to other antimicrobial drug groups can also result in resistance to trimethoprim. We found an association between the use of >3 prescriptions of extended-spectrum penicillins, such as amoxicillin, and trimethoprim resistance. Notably, we also found an association between >3 prescriptions of nitrofuran derivatives, such as nitrofurantoin, and less trimethoprim resistance, probably because nitrofurantoin killed the trimethoprim-resistant *E.coli*. These results had already been found in another study in which antimicrobial drug use had been related to AMR at GP practice level.[25] However, due to the design, this study could not exclude that the higher use of extended-spectrum penicillins was not the cause but the consequence of the higher levels of trimethoprim resistance. Since significant results in epidemiologic studies can easily be caused by bias in design and residual or unnoticed confounding, confirmation with a different design and in a different population is important. An advantage of our study was that we could use data on prescriptions and AMR on the level of the individual and that we selected on antimicrobial drug use before culturing. The fact that use of

extended-spectrum penicillins is associated with trimethoprim resistance is probably explained by the fact that the bacteria that carry genes causing trimethoprim resistance also carry genes causing resistance to extended-spectrum penicillins, selecting out these bacteria. In contrast, nitrofurantoin can kill trimethoprim-resistant bacteria, when these do not carry genes that also makes them resistant to nitrofurantoin, which is not unlikely since resistance to nitrofurantoin is low.

Diet

Next to the use of antimicrobial drug use, we found several other potential risk factors for AMR in UTIs, one of which was dietary intake. In chapter 3.1, we showed that a high intake of pork and chicken was associated with ciprofloxacin resistance. Furthermore, chapter 3.3 is completely dedicated to diet as a risk factor for AMR in UTIs caused by *E.coli*. It shows associations between a high intake of chicken and cefotaxime resistance and a higher intake of pork with norfloxacin resistance in UTIs caused by *E.coli*. This last association, of course, was similar as in the study with ciprofloxacin resistance, since norfloxacin and ciprofloxacin are both fluoroquinolones, partly similar cultures were used and resistance to both is (partly) caused by similar mechanisms. We did not find an association with chicken and norfloxacin in this population.

It has been suggested for years that antimicrobial drug use in animals could result in AMR in humans. Addressing AMR is one of the pillars of One Health, which looks for a multisectoral approach, because similar antimicrobial drugs are used in animals and humans.[26] And although many studies already tried to prove that there is in fact transmission of resistant pathogens or resistance genes from animals to humans, the subject remains highly debated and the evidence is indirect. The introduction of fluoroquinolones into broiler chicken production in the USA, for example, was associated with an increase in ciprofloxacin-resistant campylobacters in humans.[27] Additionally, only a low prevalence of fluoroquinolone resistance has been found in human samples in Australia and Finland, where the use of fluoroquinolones in food animals is prohibited.[28, 29] The studies that we performed, certainly contribute to the discussion, but also raise more questions. Diet studies in general are difficult to perform and easily subjected to bias and confounding. Furthermore, the reported use of higher generations cephalosporins and ciprofloxacin in animals in the Netherlands is low, certainly because these drugs are classified as critically important for humans and use in animals is only allowed under strict conditions.[30, 31] Fortunately, antimicrobial drug use in livestock is decreasing more and more in The Netherlands in most recent years: in 2019 antimicrobial drug use was 69% less than in 2009.[32] Furthermore, whether a resistance gene or resistant pathogen is transferred from an animal to a human being, for example by direct contact, eating meat or contaminated products, and incorporated in the microbiota is most likely dependent on a complex interplay of numerous factors. And this not even includes the fact that being a carrier of resistance does not mean that somebody will suffer from infections with this resistant pathogen.

Concomitant drug use

Next to diet, we also found that concomitant drug use could possibly result in a higher prevalence of AMR in UTIs in some cases. In chapter 3.1, we showed that both the concomitant use of calcium supplements and proton pump inhibitors (PPIs) were associated with resistance to ciprofloxacin in *E.coli* isolates. Both PPIs, possibly via an increased gastric PH,[33, 34] and calcium have been shown to decrease the bioavailability of fluoroquinolones.[35-37] The bioavailability is important since fluoroquinolones are belonging to the concentration-dependent antimicrobials, meaning that the efficacy is dependent on the ratio of the peak concentration (C_{max}) during a dosing interval and the Minimal inhibitory Concentration (MIC), which is the lowest concentration that prevents growth of the bacteria. A lower C_{max}/MIC is therefore suggested to increase the risk for resistance.[38, 39]

Target 3: The interplay between antimicrobial drugs, AMR and the gut and genitourinary microbiota

The gut microbiota is the community of microorganisms living in the intestines. The interest in the gut microbiota has been growing in the last decades, since the technical possibilities to investigate this entity have increased and because it is thought that it plays an important role in human health. Next to this, recently also the existence of (genito)urinary microbiota has been acknowledged, where it has been thought for long that the urinary tract is sterile. The gut microbiota has been suggested to be the reservoir for resistant bacteria and could therefore be important in the development of UTIs with resistant bacteria. However, the interplay between the gut and genitourinary microbiota and AMR is complex, since also the use of antimicrobial drugs itself could have an effect on the composition of both.

In chapter 4.1, we studied the association between the use of antimicrobial drugs and the genitourinary microbiota. We found an association between these two in a small study. However, we could not determine whether antimicrobial drugs change the composition of the genitourinary microbiota or whether genitourinary dysbiosis results in UTIs, which need to be treated with antimicrobial drugs.

The fact that antimicrobial drugs do have effects on the gut microbiota was already established. For example, it is known that the use of antimicrobial drugs can result in overgrowth of specific bacteria, such as *Clostridium difficile*. More recently, multiple studies have shown that antimicrobial drugs can indeed change the composition of the gut microbiota itself.[40, 41] In chapter 4.2, we studied the long-term effects of antimicrobial drugs on the gut microbiota in a large population-based cohort, comparing several antimicrobial drugs. We showed that the group of macrolides and lincosamides have strong and prolonged effects (up to 4 years after use) on both the diversity and community structure of the gut microbiota, most likely due to the

anaerobic activity of the lincosamides, such as clindamycin. We also found an association between beta-lactam antibacterials and effects on the diversity and community structure in the first year after use. A recent systematic review also showed the most prolonged effects for clindamycin (2 years), clarithromycin and metronidazole (4 years), next to ciprofloxacin (1 year).[42]

Additionally, in chapter 4.3, we studied carriership of several beta-lactamase and extended spectrum beta lactamase genes. We investigated this carriership using RT-PCR in a community-dwelling cohort with middle-aged and elderly individuals. This is in contrast to many other studies, which investigate carriership in patients using culturing techniques. We found that carriership of the extended-spectrum beta-lactamase CTX-M was rather low, whereas carriership of AmpC-producing CMY was also quite low, but not that low in proportion to carriership of CMY. Furthermore, we studied potential risk factors for carriership. Interestingly, antimicrobial drug use did not seem to be a very important factor in the risk for carriership, whereas we found some suggestions, but not a very clear role for the composition of the microbiota. The use of PPIs was associated with the presence of three of four genes. Associations between PPIs and multi-drug resistant bacteria have recently also been suggested in several other studies.[43-45] Furthermore, PPIs are also suggested to affect the gut microbiota, which might also be the reason that they are associated with *Clostridium difficile* infections.[46, 47]

Target 4: Risk factors for urinary tract infections

Since UTIs are very common infections, preventing UTIs could also be a good target in diminishing AMR. This is especially true, since some individuals suffer substantially more often from UTIs, suggesting that there must be certain risk factors that make some individuals more prone than others to develop UTIs. Well-known risk factors are sex (UTIs are more common in women than in men), age, co-morbidities, such as abnormalities of the urinary tract and diabetes, sexual activity, and also a history of UTIs. It is widely believed that UTIs are more common in women than in men, because of the shorter distance from the anus to the urethra in women. In this hypothesis, it is assumed that uropathogens originate from the gut and indeed many uropathogens, such as *E.coli* and *Klebsiella* species are known to be inhabitants of our intestines. It has already been debated for years whether recurrent UTIs are caused by a recurrence of a not adequately eradicated bacteria or by a re-infection of a new (or the same) organism from the gut.[48] This could be caused by host factors, but it could also be that these women carry for some reason more pathogenic *E.coli*. A study that used whole genome sequencing compared fecal *E.coli* and *E.coli* that caused UTIs showed a close relationship, suggesting that the uropathogenic *E.coli* could indeed originate from the gut.[49] However, another sequencing study concluded that uropathogenic *E.coli* are genotypically very diverse and that they probably diverged from non-pathogenic strains at least 100.000 years ago. This

suggests that the type of *E.coli* is important in the development of UTIs.[50] Thus, although we are several decades and many improving techniques further, we still do not understand why some women suffer from recurrent UTIs whereas others do not. We tried to add some new insights to this discussion in chapter 4.4: we studied whether the composition of the gut microbiota could play a role the development of UTIs in middle-aged and elderly women. Interestingly, we found an association between the community structure of the gut microbiota and the time to the first UTI in women without a recent UTI history. Although, this is only one study in one population, this could suggest that the gut microbiota could play a role in the initiation of a first UTI, where a specific composition might result in favorable conditions to cause an *E.coli* from the gut to migrate to the urinary tract.

Concluding remarks and remaining questions

The aim of this thesis was to find targets that can be used to diminish AMR in UTIs, in order to make sure that these infections can also be treated in the future. We elaborated on 4 possible targets: good antibiotic stewardship, risk factors for AMR in UTIs, the interplay with the gut and urogenital microbiota and risk factors for UTIs itself. However, these 4 targets comprise of several components that are mutually related. **Figure 1** is an attempt to show all the (possible) relationships that are (possibly) present between the different components. In this section, an attempt is made to explain these connections.

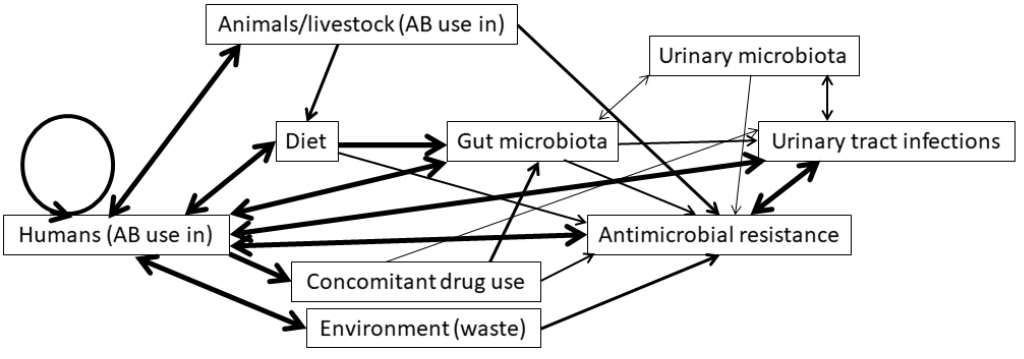

Figure 1: Graphical representation of the interactions between several important factors in this thesis. The thickness of the arrows represents the strength of the evidence of the relationships.

Use of antimicrobial drugs in humans and animals

One of the main components in the occurrence of AMR are humans. Use of antimicrobial drugs in humans directly influences AMR, but AMR also affects humans and the choice of antimicrobial drugs prescribed to humans. In chapter 2, we showed that electronic databases can be used to investigate the prescribing of antimicrobial drugs by GPs. In contrast to many other countries, in the Netherlands antimicrobial stewardship is already part of normal routine, especially in hospitals and a substantial number of antibiotic stewardship programs has been implemented. However, in our modern society with people traveling continuously to all parts of the world, we are dependent on antimicrobial stewardship worldwide. Hopefully, the recent publication of several reviews and meta-analyses, showing that antimicrobial stewardship programs were effective in reducing infections with resistant bacteria, will help in the realization of programs worldwide.[53] Although for health professionals a reduction of AMR itself could already be an important reason to implement antimicrobial stewardship programs, for decision makers cost-effectiveness of such a program is even more important. Unfortunately, a recent review concluded that there is a lack of evidence in this area. Only a few studies have investigated the cost-effectiveness of antimicrobial stewardship programs, which are mostly studying the implementation of measures in western hospitals, excluding outpatient and GP practices and non-western countries. The few studies in western hospitals suggested that antimicrobial stewardship programs could be cost-effectiveness, but the evidence was limited because the variety in study designs and measures made it hard to draw strong conclusions[54]

The One Health concept acknowledges that the health of humans, animals and the environment cannot be separated from each other.[55] Even though more research is needed to obtain firm evidence, the use of antimicrobial drugs in livestock is very likely contributing to AMR.[56] How the resistant pathogens of resistance genes are transferred from livestock to humans is still part of the debate, but possibilities are via the food chain, via direct contact or via animal waste, such as manure in the environment.[55] Since AMR does not stop at borders, we are not only dependent on the use of antimicrobial drugs in humans in other countries but also on their policies of prescribing antimicrobial drugs to animals. Therefore, the use of antimicrobial drugs in food animals should be monitored. In the Netherlands, strict regulations for prescribing antimicrobial drugs in livestock have been implemented. Unfortunately, many antimicrobial drugs are prescribed to food animals in many other countries worldwide, not only to treat infections, but also for prevention of infections or for growth promotion.[57] International collaboration is the key to fight AMR. Therefore, the Dutch Rijksinstituut voor Volksgezondheid en Milieu (RIVM) participates actively in projects of the European Union, such as the One Health European Joint Program, to combat the threat of AMR.[58]

Environment

Although, this thesis did not elaborate much on pollution, this is also a very important aspect to keep in mind. First of all, direct pollution of antimicrobial waste is assumed to play a very important role in the rise of AMR prevalence.[59] Also the WHO warns for environmental factors, such as limited access to clean water, poor sanitation and rudimentary waste management in the spread of AMR, since these factors expose millions of people to resistant pathogens and genes from fecal matter and waste from antimicrobial manufacturing.[60] Next to this, it has also been shown that other pollution, such as heavy metals could result in co-selection of AMR.[61, 62] In conclusion, also diminishing environmental pollution is a target to diminish AMR. Fortunately, some good initiatives have been raised, for example in India, one of the countries with high AMR rates, where antibiotic production facilities dump large amounts of waste into the environment.[63] The Indian government recently announced stringent standards on the concentrations of antibiotics in waste discharged by factories.[64]

Factors influencing pharmacokinetics

Furthermore, the relationship between antimicrobial drug use in humans and AMR can be influenced by other factors. For example, the use of concomitantly used drugs can be a risk factor for the development of AMR. It is likely to assume that other factors that affect the blood or possibly urinary levels of antimicrobial drugs can have an effect on AMR. Thus, next to concomitant drug use, we should also keep in mind other factors, such as age, kidney function, BMI, but also genetic variations, for example for encoding for CYP450-enzymes that degrade antimicrobial drugs or P-glycoprotein transporters that pump foreign proteins, such as antimicrobial drugs, out of tissues. These factors could all play a role in the pharmacokinetics of the drug. Fluoroquinolones were among the first antimicrobial drugs recognized in which the pharmacokinetics could influence AMR. The mutant selection window was hypothesized, which suggests that the antimicrobial drug selects for mutant bacteria with a reduced susceptibility in a specific zone of the antimicrobial drug concentration. When this hypothesis is correct, a concentration can be determined that should be reached for a certain time to eradicate all bacteria, including the least susceptible ones, the mutant prevention concentration (MPC).[65] Similar mechanisms were already proposed for vancomycin exposure and AMR to vancomycin in *S.aureus*.[65] More research is needed to investigate the importance of the MPC for the different antimicrobial drugs. In an ideal world, we know the MPC when administering the drug and can use therapeutic drug monitoring, resulting in optimal antimicrobial drug levels in the individual patient, which could help in preventing AMR.

PPIs

Interestingly and *a priori* unexpected, we found several associations between use of PPIs and resistance. Of course, we should acknowledge that we have investigated associations, not causal relationships, which therefore must be confirmed by other studies. PPIs are used for a variety of

indications. They are sold over the counter in the Netherlands and are therefore often seen as relatively safe drugs. In general, no or little debate is necessary for the indications of use according to national guidelines, but next to this, PPIs are also used for a variety of other indications, for which evidence is usually weak.[66] Additionally, it has been suggested that the withdrawal of PPIs can result in a rebound effect of acid hypersecretion.[67] Use without indication should be discouraged, since many adverse events have been associated with chronic PPI use, varying from myocardial infarction, fractures, chronic kidney disease and hypomagnesaemia.[68] Although, it must be noted that in most cases a causal relationship has not been proven, and it is suggested that the study design of many of these studies was suboptimal, possibly resulting in residual confounding.[69] Unfortunately, PPIs are more often prescribed to more vulnerable individuals, who are more at risk for all these adverse effects.[68] Furthermore, the association can be caused by protopathic bias, prescription of PPIs for symptoms related to the disease, or in combination with NSAIDs prescribed for pain relief during the disease. Nevertheless, PPI use has been suggested to be associated with several infectious diseases. Several studies have shown an association between PPI use and gastroenteritis, one of them eliminating the possibility of "healthy control" bias, which makes the presence of this association more valid.[70] Furthermore, also an association between PPI use and pneumonia have been suggested in several studies, but again residual confounding is likely and clinical relevance probably low.[69] In conclusion, PPIs might be associated with several unfavorable outcomes, such as in the development of infections and carriership of resistance genes. And although these results are sometimes highly debated because of the often unfavorable study designs, in which bias and residual cannot always be excluded, the efficacy-safety balance should be considered for each individual patient. Although, unfortunately, tPPIs are freely available over the counter in many countries.

The microbiota

When discussing the potential role of diet in AMR, we first think of the potential transfer of resistant pathogens or genes from animals to humans in the food chain. However, we also found dietary items that were associated with less AMR. When these associations are true, this could point to a role of the gut microbiota, which can change under the influence of diet, but also under the influence of antimicrobial drugs, of which we showed that they have potentially long-term effects on the gut microbiota. Furthermore, the gut microbiota is also suggested to be the keeper of resistance genes, the resistome.[71] When a specific composition of the microbiota is more prone to keep resistance genes or to even promote horizontal gene transfer, this could be an important target to minimize carriership of these genes. Anyway, the gut microbiota will be part of many debates in the coming years. It is very complex and has only received interest in the last decades. Many studies have found differences between the gut microbiota of healthy individuals and individuals with certain diseases, it is therefore without doubt that the gut microbiota does indeed play an important role in human health. Examples of diseases that are associated with differences in the composition of the microbiota are inflammatory bowel

disease, and other inflammatory diseases, such as rheumatic diseases; colorectal cancer; allergies; metabolic syndrome and also psychiatric diseases, such as dementia, Parkinson's disease, autism and psychotic disorders.[72, 73] Besides, a recent study even suggested that the composition of an individual's microbiota is a better predictor for disease than its genome.[74] Although, we have acknowledged the importance of the role of the microbiota in most recent years, there are still many things unknown. Many studies have already contributed to the current knowledge of the microbiota. However, the used methods have often varied. Furthermore, what these differences exactly are and mean is most of the time not clear. We also showed an association between the composition of the gut microbiota and the development of UTIs in women without a UTI history. We can conclude that the study of the gut microbiota certainly deserves much interest and could be a potential target of (personalized) therapy in the future. However, more research will be necessary before we will reach that point.

Where the gut microbiota has already received much interest, the interest for urogenital microbiota started later on. Less studies have been performed, and the numbers of individuals in these studies are often low. However, the studies that have been performed have already shown that there could also be associations between the composition of the microbiota and certain diseases. There are still many research questions about the composition of the urogenital microbiota that can be thought of. First of all, it would be very interesting to repeat our study that investigated the association between antimicrobial drug use and the composition of the urogenital microbiota in a larger population. Also, it would be good to elucidate the exact role of the gut microbiota in the development of UTIs. Furthermore, if we will find UTIs to be local relapses, the urogenital microbiota could also play an important role, and thus be an important target, as keeper of resistance genes for UTIs. Although, an additional question then will be how to explain the associations between diet and AMR in UTIs that we found in chapter 3.3.

Prevention of UTIs

When we know more about the relation between the composition of the gut and urinary microbiota and the development of UTIs, we can possibly find targets to prevent these infections. As an old Dutch saying says: "prevention is better than the cure", when preventing individuals from suffering from UTIs, no antimicrobial drug prescribing is needed at all. Several strategies to prevent UTIs have already been suggested. One of these is the use of probiotics, which can potentially diminish UTIs, especially in women, suffering from recurrent UTIs. However, probiotics are not the Holy Grail (yet).[75-78] A more recent approach is vaccination against uropathogenic *E.coli*. A study in mice has been performed and the results are promising.[79] However, it should be noticed that when applied to humans, the vaccination against *E.coli* might affect the human gut microbiota, since this could influence complex processes in the gut microbiota that play a role in health and disease.

Conclusion

This thesis elaborated on several targets that can be used to stop the rise of AMR in UTIs. Although, AMR is a naturally occurring phenomenon, the development of antimicrobial drugs, but especially their misuse by humans have caused a dramatic rise in the last decades. We will have to put much effort in international collaborations to promote the prudent use of antimicrobial drugs in both humans and animals worldwide and to prevent environmental pollution with waste from antimicrobial drug producing factories to turn the tide and to make sure that common infections still can be treated in future.

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Summary

Antimicrobial resistance (AMR) is defined as the ability of a disease-causing microbe to survive exposure to an antimicrobial agent. AMR is an increasing problem and is declared by the WHO as a global health threat. Factors that play a role in the emergence of AMR are use of antimicrobial drugs both in humans and in the food animal sector and international travel to and from areas with high AMR rates. Infections, in which AMR is a growing problem are urinary tract infections (UTIs). UTIs are amongst the most frequently occurring infections in humans, especially in women. UTIs are often caused by Gram-negative bacteria, such as *Escherichia coli (E.coli)*. In the Netherlands, most UTIs are treated by general practitioners (GPs) according to the national guideline, which is regularly updated. These guidelines describe the treatment of both uncomplicated UTIs, such as cystitis, as well as complicated UTIs, such as pyelonephritis. Updates of this guideline take into account the national resistance rates of most common uropathogens. It is important for GPs to follow the national guideline in order to optimally treat the patient and to prevent inappropriate use of antimicrobial drugs. This thesis elaborated on antimicrobial drug use in UTIs, risk factors for antimicrobial resistance and the potential role of the gut and genitourinary microbiota.

In **Chapter 2**, we have studied the antimicrobial drug prescribing patterns for UTIs by GPs in the period 1996 – 2014. We used the large primary care database IPCI to investigate all prescriptions for UTIs in patients of 12 years and older and compared their utilization with the national recommendations. We found that the number of prescriptions for UTIs increased during the study period as also reported by others. Prescriptions of antimicrobial drugs for UTIs were in line with the guideline, especially in women. In men, prescriptions for fluoroquinolones, such as ciprofloxacin, were the most commonly prescribed antimicrobial drugs during the complete study period, whereas it was only recommended in more recent guidelines. We concluded that databases, such as IPCI can be valuable as a tool for antibiotic stewardship.

The WHO considers fluoroquinolones as critically important antimicrobial drugs, which should be used prudently in both humans and animals. Fluoroquinolones are considered to have high resistance potential, but they are recommended as a first choice for the treatment of complicated UTIs by GPs in The Netherlands. In **Chapter 3.1**, we have investigated factors that play a role in selection of *E.coli* isolates that are resistant to ciprofloxacin in UTIs. We analyzed bacterial cultures, prescription data, basic characteristics and diet of individuals from participants of the Rotterdam Study, a prospective cohort study in a middle-aged and elderly population. We showed that the use of 2 or more fluoroquinolones was associated with ciprofloxacin resistance of *E.coli'* isolates causing UTIs, as was the use of at least one fluoroquinolone in the year before culturing. Interestingly, also a high intake of pork and chicken and the concomitant use of calcium supplements and proton pump inhibitors were associated with ciprofloxacin resistance of *E.coli* isolates in UTIs. Thus, besides use of fluoroquinolones, also factors that could influence its pharmacokinetics could influence ciprofloxacin resistance.

Another drug that has commonly been prescribed for UTIs is trimethoprim. **In Chapter 3.2**, we have studied factors that may influence selection of bacteria resistant to trimethoprim in UTIs caused by *E.coli*. We focused on the hypothesis that use of other antimicrobial drugs may select for trimethoprim resistance, this phenomenon is called co-resistance. The use of more than 3 prescriptions of sulfonamides and trimethoprim was associated with trimethoprim resistance, as was the use of more than 3 prescriptions of extended-penicillins. On the contrary, previous nitrofurantoin use was associated with a lower frequency of trimethoprim resistance, possibly because of low resistance rates to nitrofurantoin and thus low prevalence of co-resistance of trimethoprim and nitrofurantoin. Thus, because of co-resistance, use of other antimicrobial drugs can also result in trimethoprim resistance.

The results of **Chapter 3.1** suggested that dietary intake has a role in selection of antibiotic resistant bacteria. Therefore, in **Chapter 3.3**, we have studied the role of diet on AMR in UTIs. It has been suggested that the use of antimicrobial drugs in animals causes higher resistance rates in animals, but also in humans. Routes of transmission of AMR from animals to humans may be via the consumption of meat, or the consumption of crops contaminated with manure from these animals. However, no definite prove exists for these hypotheses at this moment. We have investigated the association between the intake of several food groups (meat, seafood, eggs, dairy products, crops) and resistance of *E.coli* to several antimicrobial drugs (amoxicillin, amoxicillin-clavulanic acid, trimethoprim, sulfamethoxazole-trimethoprim, first-generation cephalosporins, cefotaxime, nitrofurantoin, norfloxacin). We have found associations between a higher intake of chicken and cefotaxime resistance and a higher intake of pork and norfloxacin resistance. On the other hand, a higher intake of cheese was associated with lower resistance rates to amoxicillin and amoxicillin-clavulanic acid, suggesting that cheese might have a protective effect on carriership of antimicrobial resistance genes.

Another factor that could be of importance in AMR is the microbiota. The microbiota comprises all microorganisms living at a specific site. For instance, the gut microbiota compromises all micro-organisms that live in an individual's gut. Some of these bacteria carry resistance genes in their genome, making them resistant to specific antimicrobial drugs. These genes can be transferred between bacteria, for example via horizontal gene transfer. Furthermore, the composition of the gut microbiota can change over time influenced by a variety of factors. Thus, the presence of resistance genes can also change, the gut microbiota is therefore sometimes suggested to be the keeper of resistance genes. Therefore, in **Chapter 4**, we have studied the association of antimicrobial drug use and the composition of the gut and genitourinary microbiota and the carriership of several beta-lactamase genes in the gut microbiota.

In **Chapter 4.1**, we first studied the effect of antimicrobial drug use on the genitourinary microbiota. We investigated urinary samples from 23 individuals of the Rotterdam study. We have found that prior use of antimicrobial drugs was significantly associated with differences In

the beta-diversity of the genitourinary microbiota. However, we could not distinguish whether antimicrobial drugs have an effect on the composition of the genitourinary microbiota or that dysbiosis of the genitourinary microbiota made an individual more prone for a UTI, which was subsequently treated with antimicrobial drugs.

Next, in **Chapter 4.2**, we have investigated the effects of use of antimicrobial drugs on the gut microbiota. Macrolides and lincosamides were shown to have effects on the alpha-diversity and beta-diversity of the gut microbiota up to 4 years after use. The effects on both the alpha-diversity and beta-diversity of beta-lactams were present up to a year after use. Also, fluoroquinolones showed effects on the beta-diversity up to a year after use. Furthermore, the use of antimicrobial drugs with a high anaerobic activity shifted the Firmicutes/Bacteriodetes ratio toward Firmicutes in the first year after use, whereas the use of antimicrobial drugs without this activity shifted this ratio toward Bacteriodetes. We concluded that different antimicrobial drugs have different effects on the gut microbiota and on the presence or absence of resistance genes in the gut. In **Chapter 4.3**, we have studied the carriership of several beta-lactamase genes (TEM, SHV, CTX-M and CMY) by performing qPCRs on fecal samples from individuals of the Rotterdam Study. We found that the beta-lactamase gene TEM was carried by most individuals, followed by SHV. In this population, the carriership of extended-spectrum beta-lactamase CTX-M and the AmpC beta-lactamase in the gut was low. Use of extended-spectrum penicillins was associated with TEM carriership, and use of macrolides and lincosamides, with TEM and SHV carriership. Also, the use of proton pump inhibitors was associated with carriership of TEM, SHV and CMY and the richness and composition of the gut microbiota were associated with TEM and SHV carriership.

It has been suggested that uropathogens originate from the gut microbiota. Therefore, in **Chapter 4.4**, we have determined the link between the gut microbiota and the development of UTIs in middle-aged and elderly women of the Rotterdam Study without a recent UTI history. The beta-diversity was significantly associated with the occurrence of a first UTI, whereas the alpha-diversity was associated only when adjusted for technical covariates, but not when adjusted for use of antimicrobial drugs (not prescribed for UTIs) and PPIs. This suggests that the composition of the gut microbiota might play a role in the development of UTIs in women without a recent UTI history.

In conclusion, the development of antimicrobial drugs, and subsequent misuse by humans have caused an increase of AMR in the last decades. This thesis elaborated on several targets that can be seized to stop the rising AMR rates in UTIs. We will need to put much effort in promoting prudent use of antimicrobial drugs in both humans and animals worldwide to ensure that common infections still can be treated in future.

Samenvatting

Antibioticaresistentie wordt gedefinieerd als de eigenschap van een ziekte-veroorzakend micro-organisme om blootstelling aan een antibioticum te kunnen overleven. Antibioticaresistentie is een steeds groter wordend probleem en is door de WHO uitgeroepen als een bedreiging voor de mondiale gezondheidszorg. Factoren die een rol kunnen spelen in de ontwikkeling van antibioticaresistentie zijn onder andere het gebruik van antibiotica, zowel bij mensen als bij dieren, en internationale reizen vanuit gebieden met een hoge resistentie prevalentie. Een van de infecties, waarin antibioticaresistentie een steeds groter wordend probleem is, is de urineweginfectie (UWI) UWI's behoren tot de meest voorkomende infecties, voornamelijk bij vrouwen. UWI's worden vaak veroorzaakt door Gram-negatieve bacteriën, zoals *Escherichia coli* (*E.coli*). In Nederland worden de meeste UWI's behandeld door huisartsen volgens de nationale richtlijn, die regelmatig worden geüpdatet. Deze richtlijn beschrijft de diagnose en behandeling van zowel ongecompliceerde UWI's zoals cystitis, als ook van gecompliceerde UWI's, zoals pyelonefritis. De updates van deze richtlijn houden rekening met de gerapporteerde nationale resistentie van de meest voorkomende uropathogenen. Het is belangrijk dat huisartsen patiënten volgens de richtlijn behandelen om de optimale behandeling voor de patiënt te garanderen, maar ook om onjuist gebruik van antibiotica te voorkomen. Deze thesis gaat in op het gebruik van antibiotica bij UWI's, risicofactoren voor resistentie en de mogelijke rol van het microbioom van de darm en urinewegen bij UWI's.

In **hoofdstuk 2** hebben we het voorschijven van antibiotica voor UWI's in de periode 1996 – 2014 door huisartsen onderzocht. We hebben hiervoor de grote huisartsen database IPCI gebruikt om alle antibiotica voorschriften van patiënten van 12 jaar en ouder vast te stellen en dit vergeleken met de adviezen van de nationale richtlijnen gedurende deze periode. Het aantal antibiotica voorschriften voor UWI's werd groter gedurende de studieperiode, wat ook al is geconcludeerd in andere studies. Het voorschrijven van antibiotica was grotendeels volgens de richtlijn, vooral in vrouwen. In mannen werden fluorochinolonen, zoals ciprofloxacine, het meest voorgeschreven gedurende de hele studieperiode, terwijl het alleen een eerste-keus antibiotica was in de meer recente richtlijnen. We concludeerden dat gebruik van databases als IPCI een waardevolle methode kan zijn voor antibiotic stewardship.

De WHO heeft fluorochinolonen geplaatst op de lijst van zeer belangrijke antibiotica, welke zorgvuldig moeten worden gebruikt in zowel mensen als dieren. Resistentie tegen fluorochinolonen ontstaat relatief makkelijk, maar ciprofloxacine is wel een eerste-keus behandeling voor gecompliceerde UWI's in Nederland. In **hoofdstuk 3.1** hebben we factoren onderzocht die een rol spelen in de selectie van *E.coli* isolaten die resistent zijn voor ciprofloxacine in UWI's. We hebben urinekweken, medicatie data, algemene karakteristieken en het dieet onderzocht van deelnemers van de Rotterdam Studie, een prospectief cohort-studie van bewoners van 45 jaar en ouder van de wijk Ommoord in Rotterdam. Het gebruik van 2 of meer kuren van een fluorochinolone was geassocieerd met ciprofloxacine resistentie van *E.coli*

isolaten in urineweginfecties, net als het gebruik van een fluorochinolone in het jaar voor de kweekafname (voor de UWI). Verder bleek ook een dieet met veel varkensvlees of kippenvlees geassocieerd met ciprofloxacine resistentie en daarnaast ook het gelijktijdig gebruik van calcium supplementen of protonpompremmers met een fluorochinolone. Oftewel, naast het gebruik van fluorochinolonen kunnen ook andere factoren, bijvoorbeeld factoren die de farmacokinetiek van fluorochinolonen kunnen veranderen, een effect hebben op ciprofloxacine resistentie.

Een ander antibioticum dat vaak wordt voorgeschreven voor UWI's is trimethoprim. In **hoofdstuk 3.2** hebben we factoren onderzocht die mogelijk kunnen selecteren voor trimethoprim resistentie in UWI's. We hebben ons gefocust op de hypothese dat andere antibiotica dan trimethoprim mogelijk kunnen selecteren voor trimethoprim resistentie, dit wordt co-resistentie genoemd. Het gebruik van 3 of meer antibioticakuren uit de groep van de sulfonamiden en trimethoprim was zoals verwacht geassocieerd met trimethoprim resistentie, maar ook het gebruik van 3 of meer kuren antibiotica uit de groep van de extended-spectrum penicillines. Daar tegenover stond dat het gebruik van nitrofurantoïne juist was geassocieerd met lagere cijfers van trimethoprim resistentie, waarschijnlijk door een lage prevalentie van co-resistentie van nitrofurantoïne en trimethoprim. Dus, co-resistentie kan belangrijk zijn in selectie van trimethoprim-resistente bacteriën.

De resultaten uit **hoofdstuk 3.1** suggereerden dat ons dieet invloed kan hebben op de selectie van resistentie bacteriën. Daarom hebben we in **hoofdstuk 3.4** de rol van het dieet op de selectie voor antibiotica resistentie in UWI's onderzocht. Er is een hypothese dat het gebruik van antibiotica in dieren, bijvoorbeeld bij vee, kan zorgen voor hogere resistentie cijfers in mensen, door consumptie van vlees of door consumptie van gewassen die zijn besmet met mest van dieren. Echter, er is geen keihard bewijs voor deze hypothese op dit moment. We hebben geprobeerd bij te dragen aan dit onderzoek door de associatie tussen verschillende dieet items en antibiotica resistentie (voor amoxicilline, amoxicilline-clavulaanzuur, trimethoprim, cotrimoxazol, eerste generatie cefalosporinen, cefotaxim, nitrofurantoïne, norfloxacine) in UWI's te onderzoeken. We vonden associaties tussen een dieet rijk aan kip en cefotaxim resistentie en een dieet rijk aan varkensvlees en norfloxacine resistentie. Daar tegenover stond dat een dieet met veel kaas geassocieerd was met een lagere resistentie voor amoxicilline en amoxicilline-clavulaanzuur, wat suggereert dat kaas mogelijk een beschermend effect heeft voor dragerschap van resistente bacteriën.

Een andere factor, die mogelijk belangrijk kan zijn in antibiotica resistentie is het microbioom. Het microbioom omvat alle micro-organismen van een bepaalde (lichaams)locatie. Sommige van deze bacteriën hebben resistentie genen, waardoor ze resistent zijn tegen een of meerdere groepen van antibiotica. Deze genen kunnen worden overgedragen naar andere bacteriën via horizontale transfer van genen. Verder kan de samenstelling van het microbioom veranderen met de tijd onder invloed van vele verschillende factoren. De aan- of afwezigheid van resistentie

genen kan daarmee dus ook veranderen. Het microbioom wordt daarom wel eens de "bewaarder van resistentie" genoemd. Daarom hebben we in **hoofdstuk 4** onderzoek gedaan naar de associatie tussen antibiotica gebruik en de samenstelling van het microbioom van de darm en de urinewegen en het dragerschap van verschillende beta-lactamase resistentie genen in de darm.

Eerst hebben we in **hoofdstuk 4.1** het effect van antibiotica gebruik op het microbioom van de urinewegen bestudeerd. We hebben urinemonsters van 23 deelnemers van de Rotterdam Studie onderzocht en we vonden dat eerder gebruik van antibiotica geassocieerd was met een andere samenstelling van het microbioom. Het is met onze studie echter helaas niet vast te stellen of antibiotica een effect hebben op de samenstelling van het microbioom of dat de samenstelling van het microbioom iemand gevoelig maakt voor het ontwikkelen van een UWI, waarvoor antibiotica worden voorgeschreven.

In **hoofdstuk 4.2** hebben we vervolgens het effect van antibiotica op het darm microbioom onderzocht. Het gebruik van antibiotica, zoals macroliden en lincosamiden had een effect op zowel de diversiteit als de samenstelling van het microbioom tot 4 jaar na gebruik. Beta-lactam antibiotica en fluorochinolonen hadden een effect tot een jaar na gebruik. Daarnaast bleek dat antibiotica met veel anaerobe activiteit een effect hadden op de Firmicutes/Bacteroidetes ratio, met toename van de Firmicutes, terwijl andere antibiotica een toename van Bacteroidetes toonden. We kunnen hieruit concluderen dat verschillende (klassen van) antibiotica een verschillend effect hebben op het darm microbioom dan anderen en mogelijk ook een verschillend effect hebben op de aanwezigheid van resistentie genen. In **hoofdstuk 4.3** hebben we de aanwezigheid van bepaalde beta-lactamase resistentie genen (TEM, SHV, CTX-M en CMY) in de darm onderzocht door middel van qPCR in ontlasting van deelnemers van de Rotterdam Studie. Het meest voorkomende gen was TEM, gevolgd door SHV. Het dragerschap van de Extended-Spectrum Beta-Lactamase (ESBL) genen was laag in deze populatie. Het gebruik van extended-spectrum penicillines was geassocieerd met TEM-dragerschap en het gebruik van antibiotica uit de groep van macroliden en lincosamiden was geassocieerd met TEM- en SHV-dragerschap. Daarnaast waren ook het gebruik van protonpomp remmers en de samenstelling van het darm microbioom geassocieerd met TEM- en SHV-dragerschap.

De hypothese is dat uropathogenen uit de darmen komen. Daarom hebben we, als laatste, in **hoofdstuk 4.4** onderzoek gedaan naar de link tussen het darm microbioom en het krijgen van een UTI in vrouwen van middelbare leeftijd of ouder zonder recente UWI in de voorgeschiedenis. De samenstelling van het microbioom was significant geassocieerd met het krijgen van een UWI. Verder was de diversiteit van het microbioom geassocieerd met het krijgen van een UWI alleen wanneer deze associatie was geadjusteerd voor technische covariaten, maar niet wanneer ook het gebruik van andere antibiotica (niet voorgeschreven voor UWI's) en PPI's werden toegevoegd aan het model. Dit kan erop wijzen dat de samenstelling van het microbioom van de

darm een rol speelt in het krijgen van UWI's in vrouwen zonder een voorgeschiedenis met een recente UWI.

Concluderend, gebruik, maar ook misbruik van antibiotica heeft geresulteerd in een verhoging van de resistentie cijfers in de laatste decennia. Deze thesis beschrijft het onderzoek naar verschillende aangrijpingspunten om deze stijgende resistentie cijfers terug te dringen. We zullen veel aandacht moeten besteden aan wereldwijd verstandig antibiotica gebruik, zowel in mens als dier om ervoor te zorgen dat veel voorkomende infecties, zoals UWI's, ook in de toekomst nog goed kunnen worden behandeld.

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Curriculum vitae

Marlies Mulder werd geboren op 17-09-1986 in Delft. Na het behalen van het Gymnasium diploma ging zij Biofarmaceutische Wetenschappen studeren in Leiden. Na het behalen van haar bachelor in deze studie, is zij begonnen aan de studie geneeskunde bij het LUMC Leiden. In 2014 behaalde zij haar master diploma. Na enkele maanden gewerkt te hebben als ANIOS psychiatrie, begon zij in 2015 als inspecteur farmacovigilantie bij de Inspectie Gezondheidszorg en Jeugd, wat zij combineerde met een promotie traject bij het Erasmus MC. Onder supervisie van promotoren Annelies Verbon en Bruno Stricker deed zij onderzoek naar antibiotica gebruik en antibiotica resistentie bij urineweginfecties. In december 2018, verhuisde ze naar Maastricht om te starten met de opleiding als arts-microbioloog in het Maastricht UMC+.

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PhD portofolio

Department: Epidemiology, Erasmus Medical Center, Rotterdam
Research school: Netherlands Institute for Health Sciences (NIHES)
PhD period: 2015-2021
Promotors: Prof. dr. B.H.C. Stricker en Prof. dr. A. Verbon

Training	**ECTS**	**Year**
Msc in Clinical epidemiology – NIHES, Erasmus MC, Rotterdam	40	2015-2017
Courses		
English Biomedical Writing and Communication, Erasmus MC	3.0	2016
Scientific Integrity Course, Erasmus MC	0.3	2017
Microbiome I, Molmed, Erasmus MC	0.6	2017
Course on Molecular diagnostics XI, Molmed, Erasmus MC	1.0	2017
Metagenomics applied to surveillance of pathogens and antimicrobial resistance,	0.5	2018
Adherence to Medication (ICPE)	0.1	2018
Epidemiology of Vaccine Safety (ICPE)	0.1	2018
Effectief lezen en mindmappen	0.3	2018
Conferences		
Gut day		2015
WEON congres[b]		2016
Sure Symposium		2017
Gut day[b]		2017
Netherlands Centre for One Health symposium[a]		2018
Sure Symposium		2018
International Conference of Pharmaco-Epidemiology[a, b, b]		2018
KNVM-DMT Metagenomics symposium[a]		2018
ESCMID[a, b, b]		2019
[a] oral presentation, [b] poster presentation		
Teaching tasks		
Pharmaco-epidemiology and drug safety (NIHES)		2017
Biostatistical methods I (NIHES)		2017
Medische Geschiedenis (Erasmus MC)		2018
Coaching medical students		2016-2019