J Antimicrob Chemother 2016; **71**: 3258–3267
doi:10.1093/jac/dkw234 Advance Access publication 26 June 2016

Journal of Antimicrobial Chemotherapy

Impact of amoxicillin therapy on resistance selection in patients with community-acquired lower respiratory tract infections: a randomized, placebo-controlled study

Surbhi Malhotra-Kumar[1]*, Liesbet Van Heirstraeten[1]†, Samuel Coenen[1,2]†, Christine Lammens[1], Niels Adriaenssens[1,2], Anna Kowalczyk[3], Maciek Godycki-Cwirko[3], Zuzana Bielicka[4], Helena Hupkova[5], Christina Lannering[6], Sigvard Mölstad[7], Patricia Fernandez-Vandellos[8], Antoni Torres[8], Maxim Parizel[1], Margareta Ieven[1], Chris C. Butler[9,10], Theo Verheij[11], Paul Little[12] and Herman Goossens[1] on behalf of the GRACE study group‡

[1]Laboratory of Medical Microbiology, Vaccine & Infectious Disease Institute (VAXINFECTIO), University of Antwerp, Antwerp, Belgium; [2]Centre for General Practice, Department of Primary and Interdisciplinary care (ELIZA), University of Antwerp, Antwerp, Belgium; [3]Department of Family and Community Medicine, Medical University of Lodz, Lodz, Poland; [4]Clinical Research Associates and Consultants, Bratislava, Slovakia; [5]Institute of Microbiology, Faculty of Medicine, Comenius University Bratislava, Bratislava, Slovakia; [6]Unit of R&D, Jönköping, Sweden; [7]Department of Clinical Sciences, Lund University, Lund, Sweden; [8]Hospital Clinic of Barcelona, Barcelona, Spain; [9]Cardiff University, Cardiff, UK; [10]Nuffield Department of Primary Care Health Sciences, Oxford University, Oxford, UK; [11]University Medical Center Utrecht, Utrecht, The Netherlands; [12]University of Southampton, Southampton, UK

*Corresponding author. E-mail: surbhi.malhotra@uantwerpen.be
†These authors made an equal contribution.
‡Study group members are listed in the Acknowledgements section.

Received 15 January 2016; returned 2 April 2016; revised 9 May 2016; accepted 16 May 2016

Objectives: To determine the effect of amoxicillin treatment on resistance selection in patients with community-acquired lower respiratory tract infections in a randomized, placebo-controlled trial.

Methods: Patients were prescribed amoxicillin 1 g, three times daily ($n=52$) or placebo ($n=50$) for 7 days. Oropharyngeal swabs obtained before, within 48 h post-treatment and at 28–35 days were assessed for proportions of amoxicillin-resistant (ARS; amoxicillin MIC ≥2 mg/L) and -non-susceptible (ANS; MIC ≥0.5 mg/L) streptococci. Alterations in amoxicillin MICs and in penicillin-binding-proteins were also investigated. ITT and PP analyses were conducted.

Results: ARS and ANS proportions increased 11- and 2.5-fold, respectively, within 48 h post-amoxicillin treatment compared with placebo [ARS mean increase (MI) 9.46, 95% CI 5.57–13.35; ANS MI 39.87, 95% CI 30.96–48.78; $P<0.0001$ for both]. However, these differences were no longer significant at days 28–35 (ARS MI −3.06, 95% CI −7.34 to 1.21; ANS MI 4.91, 95% CI −4.79 to 14.62; $P>0.1588$). ARS/ANS were grouped by *pbp* mutations. Group 1 strains exhibited significantly lower amoxicillin resistance (mean MIC 2.8 mg/L, 95% CI 2.6–3.1) than group 2 (mean MIC 9.3 mg/L, 95% CI 8.1–10.5; $P<0.0001$). Group 2 strains predominated immediately post-treatment (61.07%) and although decreased by days 28–35 (30.71%), proportions remained higher than baseline (18.70%; $P=0.0004$).

Conclusions: By utilizing oropharyngeal streptococci as model organisms this study provides the first prospective, experimental evidence that resistance selection in patients receiving amoxicillin is modest and short-lived, probably due to 'fitness costs' engendered by high-level resistance-conferring mutations. This evidence further supports European guidelines that recommend amoxicillin when an antibiotic is indicated for community-acquired lower respiratory tract infections.

Introduction

Antibiotic resistance is now one of the most pressing global threats to human health according to the World Economic Forum.[1] ECDC estimated that 25000 human deaths and excess healthcare costs and productivity losses of at least €1.5 billion annually were directly attributable to antibiotic-resistant bacteria.[2] US CDC estimates are even higher, attributing a minimum of 2 million illness episodes,

23000 deaths, and healthcare and productivity losses of >US$35 billion to antibiotic resistance in the United States.[3]

Antibiotic use is widely associated with antibiotic resistance, but demonstrating causality is challenging because of population-based confounders.[4] In addition, antibiotics of the same class vary widely in effects on the selection of resistant organisms.[5,6] We previously studied the effects in volunteers administered two macrolides, azithromycin or clarithromycin, and found wide variation not only in the proportions of macrolide-resistant streptococci but also in the resistance-conferring genes and mechanisms selected by these antibiotics.[5] In addition to antibiotic use, the magnitude of the 'fitness cost' associated with a resistance mechanism impacts the survival and dissemination of the bacterium in the absence of antibiotic selective pressure.[7,8] We previously found that maintaining macrolide (and streptogramin, lincosamide and tetracycline) use below a critical threshold was associated with a low prevalence of macrolide-resistant *Streptococcus pyogenes* in Belgium and an increased proportion of the 'low-cost', macrolide-resistance conferring *erm*(A) gene in *S. pyogenes*.[8]

Amoxicillin, a β-lactam antibiotic, is recommended as first-line therapy by the European Respiratory Society for treatment of community-acquired lower respiratory tract infections (CA-LRTI), the commonest reason for patient consultations in the community.[9] Amoxicillin is therefore also the most commonly prescribed antibiotic in European primary care, accounting for an average 40% of the total outpatient antibiotic use in European countries.[10,11] However, despite this, its potential for selection of resistance in *Streptococcus pneumoniae*, the most common bacterial pathogen causing CA-LRTI,[9] and its persistence *in vivo* is not yet known. As with other β-lactams, amoxicillin affects bacterial cell wall synthesis. Resistance to β-lactams in streptococci, including *S. pneumoniae*, occurs by chromosomal alterations in cell wall synthesizing enzymes, the so-called penicillin-binding proteins (PBPs). Such alterations in PBPs are due to a continuous mutation process that causes various degrees of resistance, from reduced susceptibility through low-level resistance—conventionally termed intermediate or non-susceptibility—to full clinical resistance.

We have previously demonstrated oropharyngeal streptococci as ideal model organisms to study resistance selection in *S. pneumoniae in vivo*.[5] Here we carried out a randomized, placebo-controlled trial (RCT) to quantify the impact of exposure to amoxicillin treatment on resistance in the oropharyngeal flora of patients with confirmed CA-LRTI. Furthermore, we also studied the molecular mechanisms of resistance and associated fitness costs in streptococci that emerged under amoxicillin therapy and correlated these to the resistance trajectory.

Patients and methods

Trial design and sampling

This study is nested within an RCT, which was conducted during three winter seasons from 2007 to 2010 at 16 primary care networks in 12 European countries as part of the GRACE project (Genomics to combat Resistance against Antibiotics in CA-LRTI in Europe).[12,13] In total, 3108 adults with acute uncomplicated CA-LRTI were included in an observational study of which 2061 entered the RCT.[12,13] In each of the five geographically disseminated European networks (Antwerp, Barcelona, Bratislava, Jonköping and Lodz), a subset of practices was selected to include patients (n=102) prospectively for this study in the third winter season (2009–10). Based on sample size calculations, 50 patients were required in each of the two groups. Therefore, each network was asked to include ∼20 patients. Patients who fulfilled the eligibility criteria outlined below and were willing to provide three oropharyngeal samples (Figure 1) were included and subsequently randomly allocated to receive amoxicillin therapy (1 g, three times daily for 7 days) or the matched placebo regimen.

Patients consulting for the first time with acute uncomplicated CA-LRTI were eligible if they were aged 18 years or older, immunocompetent, not pregnant, not allergic to penicillin, provided written informed consent and had not received antibiotic treatment during the previous month. Acute uncomplicated LRTI was defined as an acute cough (≤4 weeks) as the main symptom or alternatively, if cough was not the main symptom but where the clinician considered acute LRTI was the main presenting diagnosis. Patients with a clinical diagnosis of CA-pneumonia based on focal chest signs (focal crepitations or bronchial breathing) and systemic features (high fever, vomiting, severe diarrhoea) or non-infective cause of cough (e.g. allergy, pulmonary embolus, etc.) were ineligible.[12]

Oropharyngeal samples were collected from patients by swabbing the posterior pharynx and tonsillar areas and avoiding the tongue, gingiva and teeth. Three samples were obtained from each patient: before the start of treatment (day 0); within 48 h after completion of treatment (day 8); and finally, within 28–35 days after beginning treatment (days 28–35). All swabs were stored in skimmed milk medium at −80°C[5] and transported on dry ice to the central laboratory at the University of Antwerp, Belgium.

Randomization and masking

Randomization and masking has been described previously.[12] Briefly, trial drugs were block randomized by an independent statistician. The randomization codes were sent to the manufacturer, who prepared containers with the contents (amoxicillin or placebo). Both clinicians and patients were blind to the randomization sequence, and all outcome data were gathered blind to allocation status. There was a 24 h unblinding with access to the code in the event of an emergency.

Quantification of amoxicillin-non-susceptible and -resistant streptococci

At the central laboratory, each sample was thawed, vortexed and 50 μL aliquots of a 10-fold serial dilution were spiral-plated on five plates of *Streptococcus* selective medium (Oxoid, Basingstoke, UK): one without an antibiotic supplement, two supplemented with amoxicillin at 0.5 and 2 mg/L final concentration (Sigma, Sigma-Aldrich Co., St Louis, MO, USA), and another two supplemented with penicillin at 0.25 or 2 mg/L final concentrations (Fluka; Sigma-Aldrich Co.).[5] Plates were incubated for 48 h at 37°C in 5% CO_2. Proportions of amoxicillin- and penicillin-non-susceptible (ANS: isolates growing on plates with 0.5 mg/L of amoxicillin; PNS: isolates growing on plates with 0.25 mg/L penicillin) and -resistant streptococci (ARS, PRS; isolates growing on plates with 2 mg/L of amoxicillin and penicillin, respectively) were determined by dividing the colony counts of the antibiotic-containing plate by the colony counts of the plate without antibiotics.

Elucidation of amoxicillin resistance mechanisms linked to mutational changes in PBP genes and MIC determination

Twenty patients from the amoxicillin-treated group and 10 patients from the placebo group were randomly selected from among the five networks and streptococcal isolates recovered from their samples at all three timepoints were tested for mutational changes in the conserved domains of PBP genes, and for MICs of amoxicillin. For each patient, 10 colonies isolated and subcultured (for purity) from the selective agar plate containing 2 mg/L of amoxicillin were studied. If <10 colonies grew on the amoxicillin

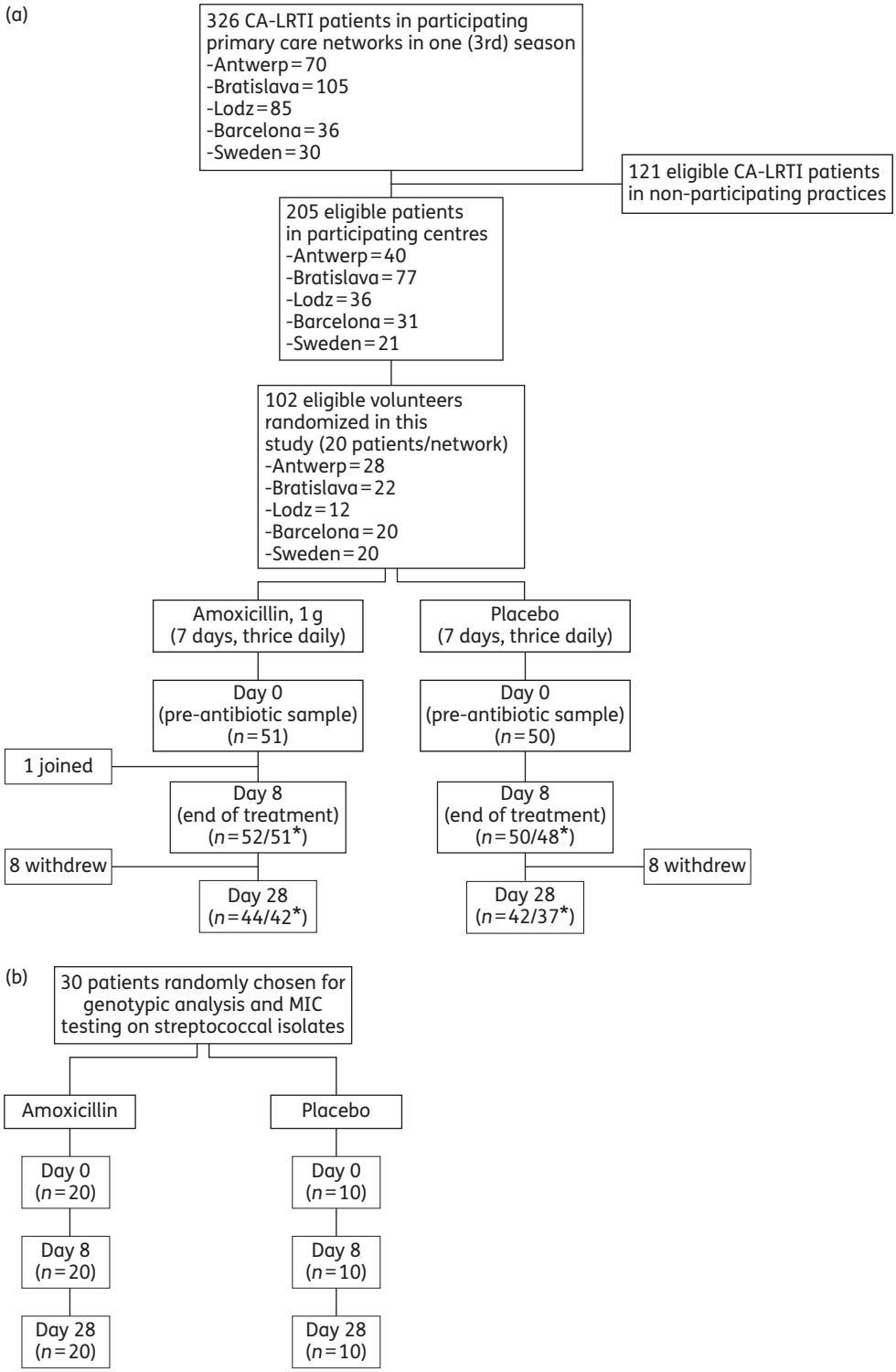

Figure 1. Trial profile. The asterisks indicate the number of patients included at days 8 and 28 for PP analysis.

(2 mg/L)-containing plates, colonies from the plates containing 0.5 mg/L amoxicillin were also included to ensure screening of 10 isolates per patient at each time-point.

Point mutations and predicted amino acid changes in PBP2x, PBP2b and PBP1a mapping to the three conserved domains [SXXK, SXN, KT(S)G] were investigated in each of the three genes: (i) PBP2x: $S_{337}TMK_{340}$, $S_{395}SN_{397}$, $K_{547}SG_{549}$; (ii) PBP2b: $S_{385}VVK_{388}$, $S_{442}SN_{444}$, $K_{614}TG_{616}$; and (iii) PBP1a: $S_{370}TMK_{373}$, $S_{428}RN_{430}$, $K_{557}TG_{559}$. For each investigated PBP gene, two primer pairs were used to amplify the SXXK and SXN regions and another for the KT(S)G region/motif. However, as oral commensal

streptococci show remarkable differences in their PBP sequences, the development of multiple degenerate primer sets ($n=16$) was required in most cases (primer sequences available on request). Amoxicillin MICs were determined for all colonies by agar dilution and isolates were classified as resistant, non-susceptible (intermediately resistant) or susceptible according to CLSI guidelines.[14]

Fitness estimations by growth and nutritional competition

The *in vitro* relative fitness of streptococci harbouring various mutations was estimated based on pair-wise competition experiments.[15,16] Briefly, overnight cultures of streptococcal strains were diluted 10-fold, grown to an OD_{600} ~0.25, mixed in a 1:1 ratio, diluted 100-fold and incubated for 6 h at 37°C with 5% CO_2. Initial and final concentrations of the competing strains were determined by spiral plating and colony counting on blood agar with and without amoxicillin (4 or 2 mg/L based on the differences in MICs of the two competing strains). The number of generations (g) grown by both strains was calculated as $g=(\log B-\log A)/(\log 2)$, where A and B are the initial and final colony-forming units (cfu)/mL of each strain. Relative fitness of each competed strain pair was determined by the ratio of the number of generations grown by both strains. A relative fitness of 1 indicates that both strains are equally fit, while a ratio ≥ 1 indicates increased or decreased fitness, respectively.[16] From each amoxicillin-containing plate, five colonies were subcultured and their genotype and MICs of amoxicillin were re-confirmed by PCR sequencing of the *pbp*2x fragment and by Etest, respectively.

Statistical analysis

A sample size of 50 volunteers in each group was needed to identify a 25% increase (60% of the standard deviation estimated at 40%) in the mean proportion of non-susceptible bacteria following antibiotic exposure with 80% power at a two-sided significance level of 5%. Data analysis was done using SPSS version 12·0 and SAS version 9.2. Differences in baseline (day 0) characteristics between the two study groups were assessed using χ^2 and t-tests (Table S1, available as Supplementary data at *JAC* Online). Means and 95% CI were used to describe changes in proportions of the ARS and ANS (or PRS/PNS) carriage, the primary outcome of our study. Similar regression methods were applied as previously reported.[5] The impact of amoxicillin use on mean proportions of ANS/ARS and PNS/PRS between and within study groups for different sampling time-points was analysed by a general linear mixed model (PROC MIXED in SAS) using the following covariates: gender, age in decades and smoking status. The secondary outcome was presence of mutations in the streptococcal *pbp* genes following amoxicillin exposure. Presence of mutations in the *pbp* genes at the level of the streptococcal colonies at two post-antibiotic sampling time-points (days 8 and 28) was compared with that at the pre-antibiotic time-point (day 0) by a generalized linear mixed model (PROC GLIMMIX in SAS) taking into account variations in the *pbp* genes observed in the placebo group. And finally, as another secondary outcome, the effect of antibiotic exposure on amoxicillin MICs among ARS/ANS was also analysed. For this, amoxicillin MIC values were $\log_{10}$ transformed and compared between and within the study groups using a general linear mixed model (PROC MIXED in SAS).

Utilizing a linear mixed model allows combining regression methods while accounting for the repeated-measures nature of the data. Its likelihood basis ensures broad validity of the model under the wide missing-at-random class of non-response mechanisms. Moreover, a person-specific random effect was introduced in the models to take into account the intra-class (intra-person) correlation. Analyses were done on an ITT basis as well as PP based on 100% adherence.

Ethics

All research sites obtained ethical and competent authority approval for this study, registered with WHO's International Clinical Trials Registry Platform (ISRCTN 52261229). Written informed consent was provided by all patients included in this study.

Results

Amoxicillin use is strongly associated with increased carriage of amoxicillin-resistant bacteria

Figure 1 shows the trial profile. One hundred and two patients with confirmed CA-LRTI were randomly assigned to receive a course of amoxicillin or a placebo for 7 days. Age, gender, smoking status and other characteristics were similar across both groups (Table 1, Table S1). One patient receiving amoxicillin did not provide the day 0 sample and 8 patients in each group dropped out after the day 8 sample for unknown reasons (Figure 1). These data were utilized for the ITT analysis. Figure 1 also shows the number of patients with 100% adherence included in the PP analysis per time-point, which reduced the number of included patients in the amoxicillin-treated group at days 8 and 28 by one and two patients, respectively, in comparison to the ITT analysed group (Figure 1). Expectedly, results obtained with both analyses were very similar (Tables 2 and 3). All results described hereunder are based on ITT analyses.

Immediately following amoxicillin therapy, mean proportions of ARS (amoxicillin MIC $\geq$2 mg/L) and ANS (amoxicillin MIC $\geq$0.5 mg/L) increased 20- and 3.5-fold, respectively, at day 8 in comparison to day 0 (9.81% and 48.21% mean increase, respectively; $P<0.0001$ for both) (Table 2 and Figure 2). Compared with the placebo group, ARS and ANS in the amoxicillin group increased 11- and 2.5-fold at day 8 (9.46% and 39.87% mean increase, respectively; $P<0.0001$ for both) (Table 3 and Figure 2). By days 28–35, differences in proportions of ARS and ANS between the amoxicillin and placebo groups were statistically non-significant ($P\geq 0.1588$), although within the amoxicillin group, mean ANS proportions remained significantly higher than at day 0 (14.41% higher; $P=0.0004$) (Tables 2 and 3, Figure 2).

In the amoxicillin-treated group, a >25% increase in ANS and ARS proportions was observed at day 8 in 78% (40 of 51) and 16% (8 of 51) patients, respectively, compared with their baseline proportions (Table 4). In contrast, in the placebo group similar

Table 1. Baseline characteristics of CA-LRTI patients

Demographic characteristics	Amoxicillin ($n=52$)	Placebo ($n=50$)
Age, years (range)	58 (25–85)	56 (20–81)
Men, *n* (%)	15 (29)	17 (34)
Smokers, *n* (%)	11 (21)	12 (24)
Prior antibiotic use in past 6 months, *n* (%)	6 (12)	7 (14)
ANS at day 0, % (95% CI)	17.9 (13.0–22.8)	24.1 (17.6–30.7)
ARS at day 0, % (95% CI)	0.5 (0.2–0.8)	0.8 (0.3–1.3)

ANS, amoxicillin-non-susceptible streptococci; ARS, amoxicillin-resistant streptococci.

Table 2. Change in mean proportions of amoxicillin- and penicillin-non-susceptible and amoxicillin- and penicillin-resistant streptococci from baseline. All data analysis shown here is ITT except for day 28 versus day 0 comparisons for the placebo group where PP analysis yielded different values (shown in italics)

	Amoxicillin[a]				Placebo[a]			
	amoxicillin-non-susceptible streptococci		penicillin-non-susceptible streptococci		amoxicillin-non-susceptible streptococci		penicillin-non-susceptible streptococci	
Time	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*
Day 0	—	—	—	—	—	—	—	—
Day 8	48.21 (40.75–55.67)	<0.0001	46.92 (39.55–54.29)	<0.0001	2.30 (−5.26 to 9.86)	0.5492	−0.57 (−8.04–6.90)	0.8806
Day 28	14.41 (6.48–22.33)	0.0004	14.93 (7.07–22.79)	0.0002	3.46 (−4.60 to 11.52)/ *1.96 (−5.84 to 9.76)*[b]	0.3983/*0.6206*[b]	5.93 (−2.06–13.92)	0.1448
	amoxicillin-resistant streptococci		penicillin-resistant streptococci		amoxicillin-resistant streptococci		penicillin-resistant streptococci	
	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*
Day 0	—	—	—	—	—	—	—	—
Day 8	9.81 (6.10–13.53)	<0.0001	5.49 (2.49–8.50)	<0.0004	0.01 (−3.64 to 3.88)	0.9506	0.19 (−2.86 to 3.23)	0.9030
Day 28	1.30 (−2.61 to 5.21)	0.5119	−0.02 (−3.39 to 2.94)	0.8900	4.13 (−0.01 to 8.12)/ *2.46 (−0.45 to 5.39)*[b]	0.0418/*0.0979*[b]	1.70 (−1.52 to 4.93)	0.2982

[a]CA-LRTI patients who received amoxicillin therapy or a placebo.
[b]PP analysis results are shown in italics.

Table 3. Difference in mean proportions of amoxicillin- and penicillin-non-susceptible and amoxicillin- and penicillin-resistant streptococci between groups. All data analysis shown here is ITT except the day 28 comparisons between the amoxicillin and placebo groups where PP analysis yielded different results

	Amoxicillin versus placebo[a]			
	amoxicillin-non-susceptible streptococci		penicillin-non-susceptible streptococci	
	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*
Day 0	−6.03 (−14.98 to 2.91)	0.1851	−2.70 (−12.61 to 7.20)	0.5914
Day 8	39.87 (30.96–48.78)	<0.0001	44.79 (34.92–54.66)	<0.0001
Day 28	4.91 (−4.79 to 14.62)/*6.72 (−3.50 to 16.93)*[b]	0.3190/*0.1958*[b]	6.29 (−4.32 to 16.92)/*12.25 (0.96–23.54)*[b]	0.2437/*0.0336*[b]
	amoxicillin-resistant streptococci		penicillin-resistant streptococci	
	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*
Day 0	−0.02 (−4.13 to 3.67)	0.9060	0.00 (−3.16 to 3.29)	0.9673
Day 8	9.46 (5.57–13.35)	<0.0001	5.37 (2.16–8.58)	0.0012
Day 28	−3.06 (−7.34 to 1.21)	0.1588	−1.86 (−5.39 to 1.67)	0.2997

[a]CA-LRTI patients who received amoxicillin therapy or a placebo.
[b]PP analysis results are shown in italics.

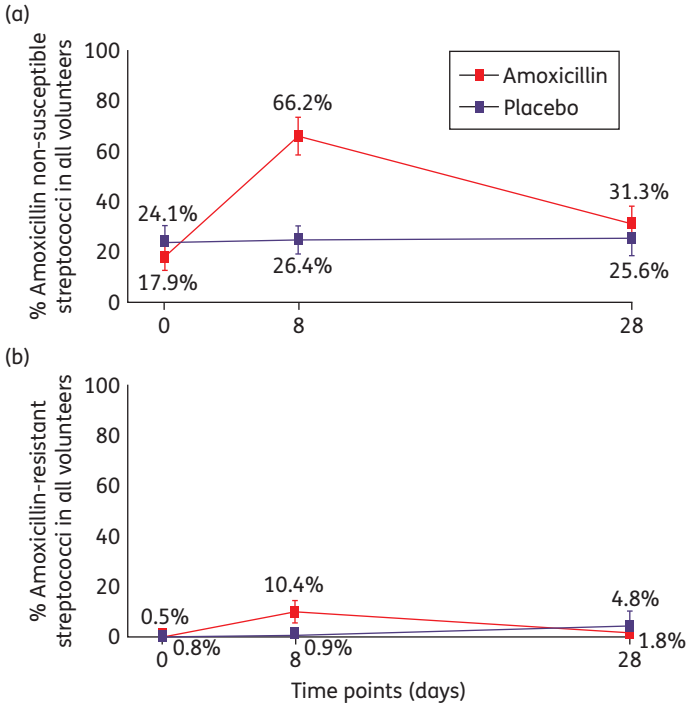

Figure 2. Temporal changes in mean proportions of amoxicillin-non-susceptible (a) and -resistant (b) streptococci after amoxicillin use. Data shown are for all 102 CA-LRTI patients followed through till days 28–35. Error bars are 95% CI.

changes in ANS and ARS proportions were only observed in 12% (6 of 50) and 0% (0 of 50) patients, respectively ($P<0.0001$). By days 28–35, the >25% increase in ANS and ARS proportions was sustained in only 26% (11 of 42) and 2% (1 of 42) of the amoxicillin-treated patients, respectively, and in 20% (8 of 41) and 5% (2 of 41) of the placebo group, respectively ($P\geq0.6028$) (Table 4).

Of note, one-third of patients treated with amoxicillin did not show any ARS in their day 8 samples ($n=18$, 34.62% patients), and in half of the patients treated with amoxicillin ($n=27$, 52% patients), ARS constituted <1.0% of the total oral streptococcal flora. In the placebo group, proportions of ANS remained notably stable over the 28–35 days studied, exhibiting barely ≈2.5% variation (Table 2). However, proportions of ARS in the placebo group showed a wider variation (4.0%) primarily due to an increase to 4.8% at days 28–35 (Table 2). These data were skewed in two patients that showed >80% proportions of ARS at days 28–35; resistance proportions of this magnitude were not even achieved in the amoxicillin-treated group immediately after therapy and were probably due to the unreported use of other β-lactams. Excluding these two patients from the analyses decreased mean ARS proportions at days 28–35 in the placebo group to 0.7%, reducing the between-sample variation in this group to ≈0.2%. Temporal changes in proportions of PNS and PRS followed the same patterns as for ANS/ARS (Tables 2 and 3, data not described further).

Amoxicillin use selects for increased carriage of streptococci harbouring PBP mutations conferring higher amoxicillin MICs and fitness costs

The conserved domains of *pbp2x*, *pbp2b* and *pbp1a* and adjoining regions were investigated among ARS and ANS isolated at the three sampled time-points from a random selection of 20 patients from the amoxicillin-treated group and 10 from the placebo group (Figure 1). In total, 616 isolates obtained from amoxicillin-treated patients ($n=412$ isolates) and from the placebo group ($n=204$ isolates) were analysed for *pbp* mutations and also screened in parallel for MICs of amoxicillin and penicillin.

In summarizing mutations detected at each time-point in streptococci isolated from participants of both groups, we

Table 4. Patients harbouring >25% higher proportions of amoxicillin-non-susceptible and -resistant streptococci following amoxicillin or placebo regimens compared with their own baseline levels and differences between groups

	Amoxicillin-non-susceptible streptococci				Amoxicillin-resistant streptococci			
Time	amoxicillin[a] [*n*/*N* (%)]	placebo[a] [*n*/*N* (%)]	difference between groups (95% CI)	*P*	amoxicillin[a] [*n*/*N* (%)]	placebo[a] [*n*/*N* (%)]	difference between groups (95% CI)	*P*
Day 0	—	—	—	—	—	—	—	—
Day 8	40/51 (78)	6/50 (12)	66% (52–81)	<0.0001	8/51 (16)	0/50 (0)	16% (6–26)	0.0058
Day 28	11/42 (26)	8/41 (20)	7% (−11–25)	0.6028	1/42 (2)	2/41 (5)	−2% (−11–6)	0.6158

[a]CA-LRTI patients who received amoxicillin therapy or placebo.

identified a pattern amongst the *pbp* mutations with a certain set almost always tending to co-occur. We divided the strains into two mutational groups on this basis (Table S2). Investigation into proportions of strains belonging to either group at the three time-points in the amoxicillin-treated group showed that >80% of the streptococci isolated at day 0 were group 1 strains. Immediately following amoxicillin treatment, group 2 strains became predominant (61.07%, $P<0.0001$), and although these had halved in proportions (30.71%) by days 28–35 as compared with day 8, proportions of group 2 strains remained significantly higher compared with the day 0 pre-antibiotic sample (18.70%, $P=0.0004$, Figure 3a). In the placebo group, proportions of group 1 and 2 mutational groups were similar at baseline (group 1, 51.32% and group 2, 48.68%) and exhibited a maximum variation of ≈15% in between time-points (Figure 3b).

In 8 of 11 (73%) amoxicillin-treated patients with only group 1 mutations in their investigated strains at baseline, group 2 mutations developed as *de novo* mutations at day 8, either in part or all of the investigated strains of the patient. Group 2 strains persisted at days 28–35 in five (63%) of these patients. In four patients of the amoxicillin group that harboured group 2 streptococci at day 0, these mutations were also present at day 8, either exclusively or in combination with group 1 streptococci. Of the three patients that harboured both group 1 and 2 streptococci at day 0, one patient carried only group 2 streptococci and two carried both group 1 and group 2 strains at day 8.

Next, we questioned whether the amoxicillin MICs of group 1 and 2 mutational groups would be substantially different. Indeed, amoxicillin MICs of group 1 strains were significantly lower (mean MIC 2.8 mg/L, 95% CI 2.6–3.1, MIC range 0.5–16 mg/L) than group 2 strains (mean MIC 9.3 mg/L, 95% CI 8.1–10.5, MIC range 0.5–64 mg/L) ($P<0.0001$). In parallel to the increased proportions of group 2 strains at day 8, mean amoxicillin MICs of strains also increased from 4 mg/L at day 0 to 9 mg/L at day 8 and decreased to 5 mg/L at days 28–35 (Figure 3a). Also noteworthy were the predicted amino acid substitutions T371A or -S in PBP1a that were present in both group 1 and 2 strains (Table S2). Amoxicillin MICs for streptococci harbouring either substitution were significantly higher than for those lacking them at any time-point analysed ($P\leq0.0002$). In the amoxicillin group, streptococci harbouring these substitutions increased from 52% (64 of 123) at day 0 to 69% (103 of 149) at day 8, and reverted to baseline level at days 28–35 (51%, 72 of 140). In the placebo group, proportions of T371S/A-harbouring

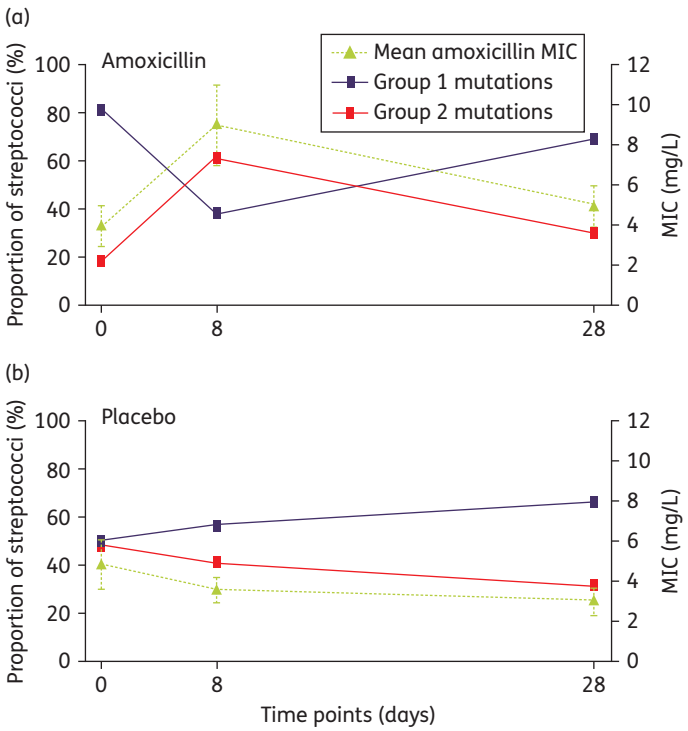

Figure 3. Temporal changes in mean amoxicillin MICs and in proportions of streptococci isolated from 30 CA-LRTI patients assigned to receive amoxicillin (a) or placebo (b).

streptococci remained constant (67%–69%) at all three time-points. Also of note, the co-occurring group 2 substitutions M339F and M400T in PBP2x were detected almost exclusively at day 8 (23 of 25, 92%) and in the amoxicillin-treated group (21 of 25, 84%). Amoxicillin MICs for streptococci harbouring these substitutions ranged between 4 and 16 mg/L. This observation made us question whether certain group 2 substitutions might be disadvantageous for the bacteria to maintain in the absence of amoxicillin pressure.

Growth competition experiments were carried out among four streptococcal strains harbouring group 1 (strain 1, recovered from a day 0 sample) and group 2 mutations (strains 2 and 3 recovered from day 8 samples and strain 4 from a day 28–35 sample)

(Table S3). Strain 1 exhibited significantly higher fitness than strains 2 and 3 (average relative fitness: 1.61 versus 0.62 and 1.49 versus 0.67; $P \leq 0.0070$, competition experiments 4 and 5) (Table S4), which were isolated at day 8 and harboured group 2 mutations (including M339F and M400T in PBP2x and strain 2 showed an additional change, M447V, in PBP2b; Tables S3 and S4). However, no significant differences in fitness were observed for strain 1 when competed with strain 4, the latter being a group 2 strain isolated at days 28–35 (competition experiment 2; Table S4).

Discussion

Utilizing oral streptococci as model organisms, we have shown that amoxicillin use is a crucial driver of resistance and non-susceptibility to amoxicillin *in vivo*. Compared with the placebo group, changes effected by amoxicillin were rather short-lived lasting ~4 weeks post-therapy. Specifically, the majority of the streptococci exhibiting high-level resistance to amoxicillin (MICs >5 mg/L) that had emerged immediately after amoxicillin therapy had disappeared within a month, most likely due to a high cost of maintaining certain resistance-conferring mutations in the chromosomal *pbp* genes in the absence of direct amoxicillin pressure.

Although proportions of ANS within the amoxicillin group remained almost double the baseline at days 28–35, proportions of both ARS and ANS showed a rapid decline upon cessation of amoxicillin treatment. These findings highlight the relatively small ecologic footprint ('collateral damage') of amoxicillin compared with the broader-spectrum macrolides, clarithromycin and azithromycin,[5] which are recommended as first-line therapy for the treatment of severe CA-LRTI (i.e. pneumonia with no risk factors for drug-resistant *S. pneumoniae* infection) in the United States and Canada.[17] We have previously shown in an RCT that a single course of either clarithromycin or azithromycin leads to a major increase in macrolide-resistant streptococci that persists for at least 6 months after therapy.[5] Taken together with the present study, our results provide strong scientific evidence in support of current European guidelines for the management of adult CA-LRTI.[9] These guidelines recommend amoxicillin as first-line therapy and that newer macrolides be reserved for patients with penicillin allergy and in regions where pneumococcal resistance to macrolides is low.[9] Resistance-related damage aside, a large, multicentre study has shown that side effects of amoxicillin therapy are about as common as the minor symptomatic benefits from amoxicillin in adults with CA-LRTI, suggesting that clinicians should prescribe amoxicillin for suspected pneumonia only rather than for all with LRTI.[12]

Our primary aim was to understand better the resistance dynamics under amoxicillin pressure of *S. pneumoniae*, the primary bacterial cause of CA-LRTI. We chose to study oral commensal streptococci as a model because common commensal species such as *Streptococcus mitis* and *Streptococcus oralis* are genetically very similar to *S. pneumoniae*,[18] and are known reservoirs of resistant *pbp* alleles or 'mosaic' genes that are easily transferred to *S. pneumoniae* during infection and subsequent β-lactam therapy.[19,20] Resistance levels in *S. pneumoniae* have been similar to levels in oral commensal streptococci in several countries, which further supports the utility of this approach.[21,22] Furthermore, the trajectory of resistant oropharyngeal streptococci (MIC ≥2 mg/L) selected by amoxicillin and their remarkable lack of persistence beyond 28–35 days is congruent with the observed Europe-wide trend of a generally low (<10%) and stable prevalence of penicillin-resistant *S. pneumoniae* despite years of heavy penicillin use (see EARS-Net Annual Report, 2011).[10] None the less, any direct comparison between *in vivo* resistance emergence at the individual level and community-wide resistance trends requires cautious interpretation.[23] Penicillin-resistant *S. pneumoniae* trends are likely influenced by the presence of multi-drug resistance in prevalent strains and co-selection by different antibiotic classes,[24] as well as by the introduction of the pneumococcal vaccine.[25] Interestingly, the type of β-lactam(s) being consumed in a particular region might also influence resistance. For example, there is a low and stable prevalence of penicillin-non-susceptible *S. pneumoniae* in the UK (5.4%), a country with a high consumption of amoxicillin [on average 4.58 DDD per 1000 inhabitants per day (DID), 1997–2009; EARSS Annual Report, 2011].[10] However, France is also one of the major consumers of amoxicillin (9.0 DID, 1997–2009) and shows one of the highest prevalences of penicillin-non-susceptible *S. pneumoniae* in Europe (23.8% of invasive *S. pneumoniae*; EARS-Net Annual Report, 2011).[10] These resistance levels might have been further influenced by an unusually high outpatient use of third-generation oral cephalosporins such as cefixime in France (but not in the UK), which has been associated with a 2.2-fold higher likelihood of carriage of β-lactam-resistant *S. pneumoniae* compared with penicillin or no antibiotic use ≤30 days after therapy.[26,27]

Amoxicillin achieves high respiratory tissue concentrations and has a short elimination half-life (≈1.2 h), which may explain the reduced risk of resistance selection in bacteria inhabiting the respiratory tract. However, the 'optimal' dose and duration of amoxicillin therapy for CA-LRTI that could further minimize emergence of resistance remains controversial. National recommendations vary widely between European countries. In the UK, a 500 mg, three times daily regimen for 5–7 days is generally recommended for CA-LRTI, whereas in Belgium, a 1 g, three times daily regimen for ≥7 days is recommended. In the present study, patients were allocated a high-dose (1 g, three times daily) 7 day amoxicillin course based on a Monte Carlo simulation aiming to reach MIC of ~1.5 mg/L to cover both *Haemophilus influenzae* and intermediately resistant pneumococci (N. Frimodt-Møller, unpublished results). This regimen might have facilitated a decreased resistance selection in our study, although previous studies investigating emergence of resistance in select patient populations administered amoxicillin in lower doses and shorter regimens have also shown similar results. Chardin *et al*. administered 1 g, twice daily amoxicillin for 3 or 7 days and did not find any differences in the proportions of non-susceptible streptococci selected after therapy (≈25%) or in those that persisted slightly above baseline levels at 30 days post-therapy.[28] A single 3 g dose did not select for any resistant streptococci,[29] while three or four 3 g doses given at weekly intervals led to a significant increase in numbers of resistant streptococci,[29] which exhibited MICs ≥2 and 40 mg/L for a period of 28 and 21 days, respectively.[29] In another study, a two-dose 3 g amoxicillin regimen given five times at weekly intervals in 12 healthy volunteers led to the emergence of resistant streptococci in saliva within 24 h of the first amoxicillin administration that declined to

undetectable levels within 13 weeks after the last amoxicillin dose.[30] A single 3 g amoxicillin dose in 10 volunteers did not result in any emergence of resistant streptococci.[31] Finally, a study comparing amoxicillin 1 g twice daily regimen for 7 days versus clarithromycin combined with omeprazole and metronidazole in patients with *H. pylori* infection recovered streptococci from the saliva of patients that were primarily non-susceptible to amoxicillin and a few with MICs of 2–4 mg/L.[32] These promising results prompt larger and more detailed studies to test the effect of more compliance-friendly amoxicillin regimens on clinical efficacy and resistance selection.

Finally, we also observed fitness deficits on pairwise competition experiments in some strains harbouring resistance-conferring mutations in *pbp* genes. These group 2 strains were isolated at day 8, harboured both M339F and M400T substitutions, and exhibited amoxicillin MICs of 4 and 16 mg/L. Changes at positions 339 and 400 in PBP2x affect the active site chemistry and are known to confer high-level resistance to β-lactams in *S. pneumoniae*.[30,33] Also remarkable was that group 2 strains with the predicted 339 and 400 substitutions could only be detected at day 8. By days 28–35, these mutants had either disappeared or were present in such low numbers that they were missed on the resistant colony screens. Their lack of persistence *in vivo* once amoxicillin selection pressure had waned could be explained by the significant fitness deficits observed on *in vitro* pairwise competition experiments. These mutants were clearly outcompeted by a group 1 strain isolated at day 0. In contrast, we did not detect any differences in fitness between the group 1 strain from day 0 and the 'persisting' group 2 strain isolated from the day 28–35 sample that also showed the highest amoxicillin MICs among the four competed strains. While negative *in vitro* results do not exclude the possibility of fitness costs under *in vivo* conditions (*vis-à-vis* positive *in vitro* results that allow a reasonable assumption that under natural conditions the resistance would engender a fitness deficit to the bacterium),[31] there might also be other explanations for this phenomenon. If some of the resistance-conferring mutations in the group 2 strain affect the housekeeping function of the PBP protein, compensatory mutations might have been selected elsewhere in the genome. For instance, the M343T substitution present in group 2 strains is not associated with amoxicillin resistance and is postulated to be a compensatory change.[32] Such mutants despite exhibiting high-level resistance are not likely affected by a decrease in amoxicillin consumption although in the long term they might potentially be outcompeted by amoxicillin-susceptible strains.

In the present study, we did not address the impact of amoxicillin use on gastrointestinal flora. This is important as most urinary tract infections and associated Gram-negative septicaemias arise from infection with organisms from the gastrointestinal tract, which will also be affected by oral antibiotic consumption. We have also not considered the clinical effects of carriage of resistant commensal streptococci. However, recent antibiotic use is the most important predictor of infections with a resistant compared with susceptible respiratory and urinary tract infection.[34,35] Even in primary care, antibiotic-resistant infections remain symptomatic for longer periods and increase burden on the health services.[36]

In conclusion, we have clearly defined the impact of amoxicillin use on resistance selection and have shown that persistence of resistance selection is significantly shorter following amoxicillin use compared with the newer macrolides, azithromycin and clarithromycin. We believe these findings provide a strong evidence base supporting: (i) European clinical guidelines that recommend prescribing amoxicillin when an antibiotic is indicated for CA-LRTI;[9] (ii) clinical prescribing of an antibiotic with a lower ecological impact such as amoxicillin when an antibiotic needs to be prescribed; and (iii) future antibiotic policy making for respiratory tract infections.

Acknowledgements

Part of this work was presented at the 51st ICAAC, Chicago, USA, 17–20 September 2011 (L-1579).

Samples were collected as part of the GRACE (Genomics to combat resistance against antibiotics in CA-LRTI in Europe) project. We thank the GPs, the GRACE study team and the patients for taking part in this study. Key members of the GRACE consortium trial team whose hard work has made this study possible are listed in the next section.

Members of the GRACE study group

Niels Frimodt-Møller, Pascale Bruno, Iris Hering, Marieke Lemiengre, Katherine Loens, Bo-Eric Malmvall, Magdalena Muras, Nuria Sanchez Romano, Matteu Serra Prat, Igor Svab, Jackie Swain, Paolo Tarsia, Frank Leus, Robert Veen and Tricia Worby.

Funding

The GRACE (Genomics to combat Resistance against Antibiotics in CA-LRTI in Europe) project is supported by the European Union FP6, contract no. LSHM-CT-2005-518226 (www.grace-lrti.org), and the TheraEDGE (an integrated platform enabling theranostic applications at the point of primary care) project is supported by European Union FP7, contract no. ICT-2007-216027 (www.theraedge.org/). The work was also supported by Research Foundation-Flanders, Belgium (grant G.027408N; www.fwo.be).

The sponsor had no role in study design, data collection, data analysis, data interpretation, or writing the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Transparency declarations

None to declare.

Author contributions

This study was designed by S. M-K., S. C. and H. G. The larger GRACE trial was designed by C. C. B., T. V. and P. L., and sampling protocols by M. I., N. A., A. K., M. G-C., Z. B., H. H., C. Lannering, S. M., P. F-V., A. T. and T. V. The day-to-day management at study sites was supervised by P. L. Experimental work was done by L. VH., C. Lammens and M. P. Data were analysed by S. M-K., L. VH., and S. C. Statistical analysis was performed by S. C. The manuscript was drafted by S. M-K., L. VH., S. C. and H. G., and was reviewed by all authors.

Supplementary data

Tables S1–S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 World Economic Forum. Insight report: Global Risks 2013, Eighth edition. 2013. http://www3.weforum.org/docs/WEF_GlobalRisks_Report_2013.pdf.

2 ECDC/EMEA Joint technical report. The bacterial challenge: time to react. 2009 Sep 17. http://ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

3 US CDC. Antibiotic resistance threats in the United States, 2013. http://www.cdc.gov/drugresistance/threat-report-2013/.

4 Steinke D, Davey P. Association between antibiotic resistance and community prescribing: a critical review of bias and confounding in published studies. *Clin Infect Dis* 2001; **33** Suppl 3: S193–205.

5 Malhotra-Kumar S, Lammens C, Coenen S *et al*. Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. *Lancet* 2007; **369**: 482–90.

6 Goossens H, Ferech M, Vander SR *et al*. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 2005; **365**: 579–87.

7 Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010; **8**: 260–71.

8 Malhotra-Kumar S, Mazzariol A, Van HL *et al*. Unusual resistance patterns in macrolide-resistant *Streptococcus pyogenes* harbouring erm(A). *J Antimicrob Chemother* 2009; **63**: 42–6.

9 Woodhead M, Blasi F, Ewig S *et al*. Guidelines for the management of adult lower respiratory tract infections—full version. *Clin Microbiol Infect* 2011; **17** Suppl 6: E1–59.

10 Versporten A, Coenen S, Adriaenssens N *et al*. European Surveillance of Antimicrobial Consumption (ESAC): outpatient penicillin use in Europe (1997-2009). *J Antimicrob Chemother* 2011; **66** Suppl 6: vi13–vi23.

11 Adriaenssens N, Coenen S, Versporten A *et al*. European Surveillance of Antimicrobial Consumption (ESAC): outpatient antibiotic use in Europe (1997-2009). *J Antimicrob Chemother* 2011; **66** Suppl 6: vi3–12.

12 Little P, Stuart B, Moore M *et al*. Amoxicillin for acute lower-respiratory-tract infection in primary care when pneumonia is not suspected: a 12-country, randomised, placebo-controlled trial. *Lancet Infect Dis* 2013; **13**: 123–9.

13 Little P, Stuart B, Francis N *et al*. Effects of internet-based training on antibiotic prescribing rates for acute respiratory-tract infections: a multinational, cluster, randomised, factorial, controlled trial. *Lancet* 2013; **382**: 1175–82.

14 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing. Twentieth informational supplement M100-S20.* CLSI, Wayne, PA, USA, 2010.

15 Van HL, Coenen S, Lammens C *et al*. Antimicrobial drug use and macrolide-resistant *Streptococcus pyogenes*, Belgium. *Emerg Infect Dis* 2012; **18**: 1515–8.

16 Rozen DE, McGee L, Levin BR *et al*. Fitness costs of fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2007; **51**: 412–6.

17 Mandell LA, Wunderink RG, Anzueto A *et al*. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin Infect Dis* 2007; **44** Suppl 2: S27–72.

18 Donati C, Hiller NL, Tettelin H *et al*. Structure and dynamics of the pangenome of *Streptococcus pneumoniae* and closely related species. *Genome Biol* 2010; **11**: R107.

19 Dowson CG, Hutchison A, Brannigan JA *et al*. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Proc Natl Acad Sci USA* 1989; **86**: 8842–6.

20 Potgieter E, Chalkley LJ. Relatedness among penicillin-binding protein 2b genes of *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus pneumoniae*. *Microb Drug Resist* 1995; **1**: 35–42.

21 Doern GV, Ferraro MJ, Brueggemann AB *et al*. Emergence of high rates of antimicrobial resistance among viridans group streptococci in the United States. *Antimicrob Agents Chemother* 1996; **40**: 891–4.

22 Potgieter E, Carmichael M, Koornhof HJ *et al*. In vitro antimicrobial susceptibility of viridans streptococci isolated from blood cultures. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 543–6.

23 Schechner V, Temkin E, Harbarth S *et al*. Epidemiological interpretation of studies examining the effect of antibiotic usage on resistance. *Clin Microbiol Rev* 2013; **26**: 289–307.

24 Baquero F, Baquero-Artigao G, Canton R *et al*. Antibiotic consumption and resistance selection in *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2002; **50** Suppl C: 27–38.

25 Huang SS, Platt R, Rifas-Shiman SL *et al*. Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* 2005; **116**: e408–e13.

26 Versporten A, Coenen S, Adriaenssens N *et al*. European Surveillance of Antimicrobial Consumption (ESAC): outpatient cephalosporin use in Europe (1997-2009). *J Antimicrob Chemother* 2011; **66** Suppl 6: vi25–vi35.

27 Samore MH, Lipsitch M, Alder SC *et al*. Mechanisms by which antibiotics promote dissemination of resistant pneumococci in human populations. *Am J Epidemiol* 2006; **163**: 160–70.

28 Chardin H, Yasukawa K, Nouacer N *et al*. Reduced susceptibility to amoxicillin of oral streptococci following amoxicillin exposure. *J Med Microbiol* 2009; **58**: 1092–7.

29 Woodman AJ, Vidic J, Newman HN *et al*. Effect of repeated high dose prophylaxis with amoxycillin on the resident oral flora of adult volunteers. *J Med Microbiol* 1985; **19**: 15–23.

30 Nagai K, Davies TA, Jacobs MR *et al*. Effects of amino acid alterations in penicillin-binding proteins (PBPs) 1a, 2b, and 2x on PBP affinities of penicillin, ampicillin, amoxicillin, cefditoren, cefuroxime, cefprozil, and cefaclor in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant pneumococci. *Antimicrob Agents Chemother* 2002; **46**: 1273–80.

31 Andersson DI, Levin BR. The biological cost of antibiotic resistance. *Curr Opin Microbiol* 1999; **2**: 489–93.

32 Stanhope MJ, Lefebure T, Walsh SL *et al*. Positive selection in penicillin-binding proteins 1a, 2b, and 2x from *Streptococcus pneumoniae* and its correlation with amoxicillin resistance development. *Infect Genet Evol* 2008; **8**: 331–9.

33 Chesnel L, Pernot L, Lemaire D *et al*. The structural modifications induced by the M339F substitution in PBP2x from *Streptococcus pneumoniae* further decreases the susceptibility to β-lactams of resistant strains. *J Biol Chem* 2003; **278**: 44448–56.

34 Costelloe C, Metcalfe C, Lovering A *et al*. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. *BMJ* 2010; **340**: c2096.

35 Hillier S, Roberts Z, Dunstan F *et al*. Prior antibiotics and risk of antibiotic-resistant community-acquired urinary tract infection: a case–control study. *J Antimicrob Chemother* 2007; **60**: 92–9.

36 Butler CC, Hillier S, Roberts Z *et al*. Antibiotic-resistant infections in primary care are symptomatic for longer and increase workload: outcomes for patients with *E. coli* UTIs. *Br J Gen Pract* 2006; **56**: 686–92.