Respiratory tract infection: prevention, early detection and attenuation of immune response

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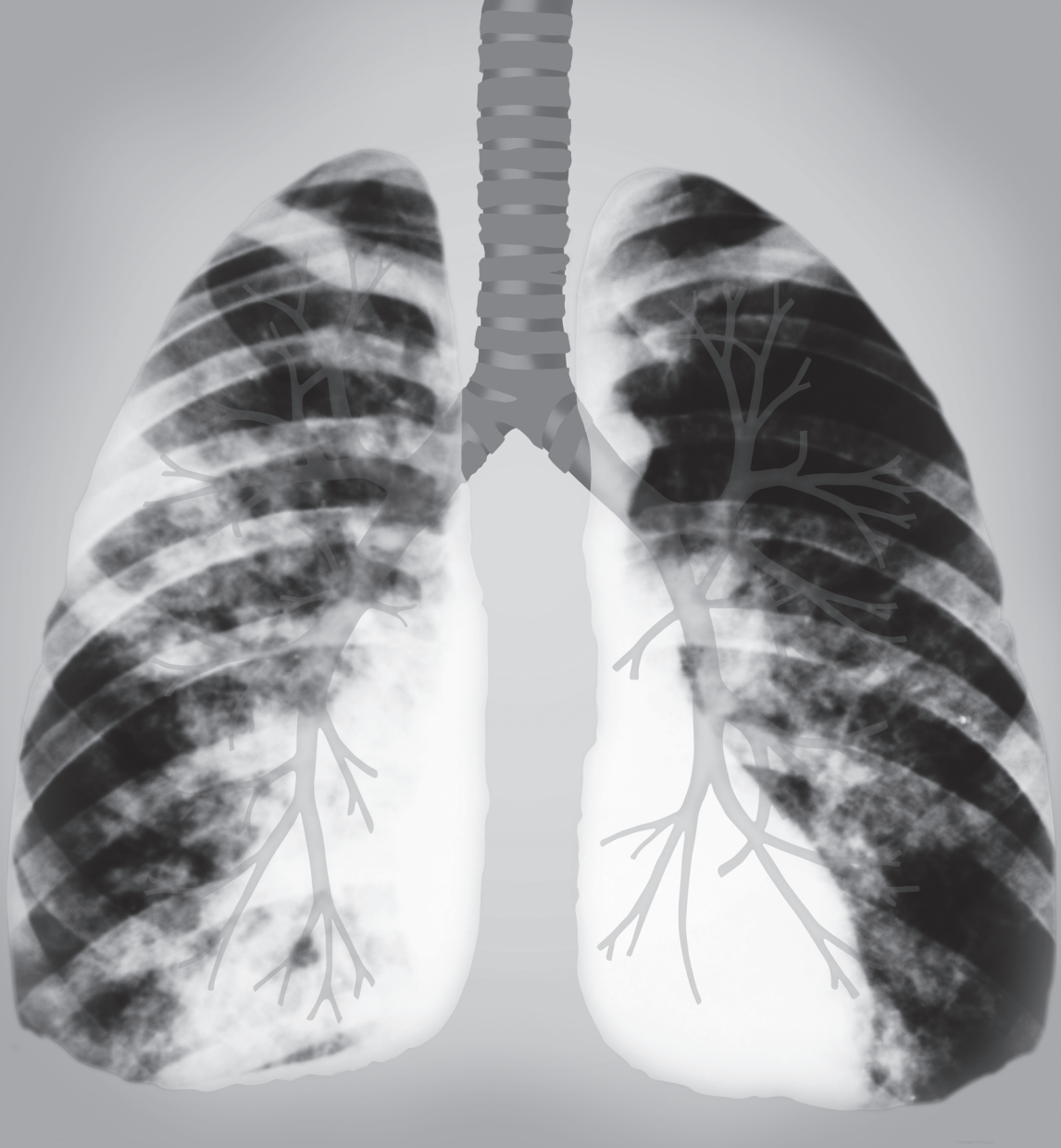

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Non-lytic antibiotic treatment in community-acquired pneumococcal pneumonia does not attenuate inflammation: the PRISTINE trial.

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ABSTRACT

Background

The inflammatory response in pneumococcal infection is primarily driven by immunoreactive bacterial cell wall components (lipoteichoic acid, LTA). An acute release of these components occurs when pneumococcal infection is treated with β-lactam antibiotics. We hypothesize that non-lytic rifampicin compared to lytic β-lactam antibiotic treatment would attenuate the inflammatory response in patients with pneumococcal pneumonia.

Methods

In the PRISTINE Trial (**P**neumonia treated with **RI**fampicin a**T**tenuates **IN**flammation), a randomized, therapeutic controlled, exploratory study in patients with community-acquired pneumococcal pneumonia, we compare LTA release, inflammatory and clinical response during treatment with both rifampicin and β-lactam compared to treatment with β-lactam antibiotics only (trial number NTR3751).

Results

Forty-one patients with community-acquired pneumonia were included, 17 of them had pneumococcal pneumonia. LTA release, LTA mediated inflammatory response, clinical outcome, inflammatory biomarkers and transcription profiles are not different between treatment groups.

Conclusions

The PRISTINE study demonstrated the feasibility of adding rifampicin to β-lactam antibiotics in the treatment of community-acquired pneumococcal pneumonia but, despite solid *in vitro* and experimental animal research evidence, failed to demonstrate a difference in plasma LTA concentrations, subsequent inflammatory and clinical responses. Most likely, an inhibiting effect of human plasma contributes to the low immune response in these patients. In addition, LTA plasma concentration could be too low to mount a response via TLR2 *in vitro*, but may nonetheless have an effect *in vivo.*

INTRODUCTION

The host inflammatory response in pneumococcal disease contributes significantly to morbidity and mortality.[1] As in other infections with Gram-positive bacteria, the inflammatory response in pneumococcal infection is primarily driven by immunoreactive bacterial cell wall components (lipoteichoic acid) or release of intracellular proteins.[2] Lipoteichoic acid (LTA) is recognized by Toll-like receptor 2 (TLR2), a pattern recognition receptor on macrophages. Binding of LTA to TLR2 induces the release of proinflammatory cytokines (e.g., IL-1, IL-6, TNF) and neutrophil influx.[3,4] Bacterial cell wall components are released when bacteria are killed by autolysis or host immune cells and are important determinants of the severity of inflammation.[5] An acute break down of bacterial cell wall occurs upon exposure to β-lactam antibiotics,[6] the first-line treatment for pneumococcal infections in many guidelines.[7,8]

Reduction of release of bacterial cell wall products may decrease inflammation, reduce tissue damage, and ultimately, reduce morbidity and mortality. Strategies to dampen the host inflammatory response have been studied extensively. Currently, dexamethasone adjunctive treatment in patients with pneumococcal meningitis is used in high-income countries to diminish inflammatory responses and consequently, neurologic sequelae.[9] In community acquired pneumonia, macrolides seem to have an immune modulatory effect by enhancement of the antibacterial effect of neutrophils and by quashing the immune response after bacterial killing.[10] However, in a clinical trial β-lactam monotherapy was non-inferior to macrolide with β-lactam combination therapy.[11]

Another potential approach is to kill the bacteria without immediately lysing them thus preventing release of proinflammatory cell wall products.[12] This would reduce the complete inflammatory trigger by interfering at the beginning of the inflammation cascade.

β-lactam antibiotics disrupt the bacterial cell wall causing lysis of the bacterium and subsequent inflammatory response. A non-lytic antibiotic such as rifampicin causes much less inflammation.[13,14] As an example, *in vitro* studies showed that rifampicin results in less release of LTA and pro-inflammatory compounds from *Streptococcus pneumoniae* than the β-lactam antibiotics ceftriaxone or meropenem, despite similar bacterial killing effects.[14] Furthermore, rifampicin may reduce inflammatory response by downregulating expression of proinflammatory pattern recognition receptors.[15] The killing of *S. pneumoniae* commences instantly after achieving therapeutic drug concentrations. Therefore, rifampicin induced non-lytic killing should start before β-lactam lytic killing.

Although animal models suggest a beneficial effect of rifamycins in the reduction of inflammation during pneumococcal infections,[13] data in humans are not available. Therefore, we hypothesize that non-lytic rifampicin compared to lytic β-lactam antibiotic treatment would attenuate the inflammatory response in patients with pneumococcal pneumonia, shortly after start of treatment.

PATIENTS AND METHODS

The PRISTINE Trial (**P**neumonia treated with **RI**fampicin a**T**tenuates **IN**flammation) is a randomized, therapeutic controlled, exploratory study in patients with community-acquired pneumonia to compare inflammatory responses during treatment with both rifampicin and β-lactam compared to treatment with β-lactam antibiotics only. The study was conducted at the Leiden University Medical Center (LUMC), a tertiary university hospital in the Netherlands. The study was approved by the LUMC Medical Ethical Committee and all patients provided written informed consent. This study was performed in compliance with the Declaration of Helsinki. The trial is registered in the Dutch trial registry, number NTR3751 (EudraCT number 2012-003067-22).

Patients

Patients were recruited at the emergency department. Inclusion criteria were:

- ≥ 18 years of age, and
- hospital admission for community-acquired pneumonia, and
- moderate to severe disease as defined by a CURB-65 score ≥2,[16] or
- one or more of the risk factors for having pneumococcal pneumonia, i.e. pleuritic chest pain, acute onset of symptoms, cardiovascular disease, leukocyte count > 15 x10e9/l, and an alveolar pattern (lobar, segmental or sub-segmental infiltrate) on chest X-ray.[17]

Exclusion criteria were: allergy to rifampicin, rifampicin-induced haemolytic anaemia or thrombopenia in medical history, liver failure, use of voriconazole or protease inhibitors, and pregnancy or breastfeeding.

Treatment

All patients were treated according to the current guidelines in the Netherlands, including at least a β-lactam antibiotic. Since resistance of *S. pneumoniae* to penicillin is extremely rare in the Netherlands,[18] empirical therapy is usually initiated with benzylpenicillin.

Patients were randomized (2:1) between the intervention group and the control group, using a prepared single randomization list. This list is generated and study patients are

assigned by independent persons. Since blinding of rifampicin treatment (with orange secretions) is impossible, this study was open label. The intervention group was treated with rifampicin 600 mg q12h intravenously for 48 hours, in combination with a β-lactam antibiotic. Rifampicin was to be given before the β-lactam antibiotic. β-lactam antibiotic treatment had to be added to the intervention treatment since this is prescribed in current guidelines, and because rifampicin resistant mutants readily appear with rifampicin monotherapy.[19] The control group was treated with a β-lactam antibiotic (without rifampicin).

In severe community-acquired pneumonia (CURB-65 score >2) or in patients with risk factors for Legionella pneumonia, ciprofloxacin is added to the empirical treatment (of patients in either group) to cover *Legionella* infection. This decision and total treatment duration was assigned by the treating physician, according to the Dutch guideline.[20]

Clinical assessment and microbiology

The clinical response was assessed by the research team using the time to clinical stability score and by monitoring the time to defervescence. Thirty and 90 days after start of therapy clinical recovery was assessed by the clinical research team.

Time to clinical stability is defined as the days from admission until: the temperature is ≤ 37.8°C, heart rate is ≤ 100 beats per minute, respiratory rate is ≤ 24 per minute, oxygen saturation ≥ 90%, systolic blood pressure is ≥90 mmHg, mental status is normal, and there is ability for oral intake.[21] If these criteria are not all met on the day of discharge, the day after discharge is defined as the day of clinical stability. Time to defervescence was defined by body temperature < 37.5°C during two consecutive measurements at least eight hours apart. The prescription of antipyretics was not part of the study protocol.

The decision to discharge a patient was left to the attending physician. Criteria to discharge were: recovery of the patient up to the level of being able to take care of themselves, and ability to complete at minimum a five day course of oral antibiotics.

Sputum culture, blood culture, nasopharyngeal swab for viral PCR, BinaxNow pneumococcal urinary antigen test, and a urinary inhibition multiplex immunoassay (IMIA) to detect and serotype pneumococci were performed to identify the causative agents.[22,23] Pneumococcal infection was defined as positive sputum or blood culture with *S. pneumoniae* or a positive BinaxNow or IMIA at inclusion.

At inclusion, at 2, 4, 8, 16, 24, and 48 hours and at 30 days after inclusion a blood sample was taken to determine the TLR2 response and to assay biomarkers. At inclusion, 24

hours, and 30 days after inclusion, blood was collected in PAXgene RNA tubes for multiplex ligation-dependent probe amplification (MLPA) assessment of inflammatory response.[24]

Outcomes

In this exploratory study, primary outcome was the feasibility of adding rifampicin to β-lactam antibiotics in the treatment of community-acquired pneumococcal pneumonia and the difference in LTA release between patients treated in the intervention group versus the ones in the control group. Secondary outcome variables are LTA mediated inflammatory response, clinical response, MLPA results and inflammatory biomarkers. Laboratory procedures to determine LTA response and LTA mediated inflammatory response are described in the supplementary data.

Clinical outcome parameters were: time to clinical stability, time to defervescence, in-hospital mortality and 30-day and 90-day mortality, length of stay in hospital and ICU admission.

Biomarker assessment

The biomarkers C-reactive protein (CRP), procalcitonin (PCT), and midregional pro-adrenomedullin (MR-proADM) were used to define inflammatory responses.[25]

CRP is measured via turbidimetric reaction with antibody-antigen complex (Roche®, Mannheim, Germany, catalogue number 12000951/12000953/04956923190). PCT and MR-proADM were determined with immunofluorescence with Time Resolved Amplified Cryptate Emission (TRACE) technology (Brahms Kryptor®, Hennigsdorf, Germany, catalogue number 82591/82592/825050 for PCT and 82991/82992/829050 for MR-proADM).

In case patients were discharged, blood sampling and biomarker assessment stopped. With clinical recovery we assumed biomarker normalization. To compensate for the missing values, the known half-lives of the biomarkers were applied (with normal value as minimum) to the last measured samples. For CRP, half-life is 19 hours (normal value 1 mg/L), for PCT half-life is 30 hours (normal value 0.15 ng/mL) and for MR-proADM half-life is 4 hours (normal value 0.36 nmol/L).

Difference in biomarkers is defined as a change of value in the first and second 24 hours after the start of treatment.

Multiplex ligation-dependent probe amplification (MLPA)

The dual-color reverse-transcriptase multiplex ligation-dependent probe amplification (dcRT–MLPA) permits accurate RNA expression profiling of 80 selected transcripts to iden-

tify biomarker signatures for host inflammatory responses to infection.[24] A Partial Least-Squares Discriminant Analysis (PLS-DA) was performed to identify components which can discriminate between groups at time point 24 hours. Variable Importance in Projection (VIP) scores is a measure of a variable's importance in the PLS-DA model. The marker with the highest VIP score is the best discriminator.

Statistical analysis

This study was an exploratory study determining the feasibility of adding rifampicin to the standard antibiotic treatment of patients with acute community-acquired pneumonia. As such, the analysis was limited to descriptive statistics and no statistical significance between groups was sought after, and by consequence, no formal power calculation was done.

Continuous variables were summarized as either means with standard deviations or medians with interquartile ranges. T-test or Mann Whitney U test was used as appropriate. Categorical variables were depicted as numbers with percentages, and Chi-squared test or Fisher's exact test was used for hypothesis testing.

To model the effect of LTA release and biomarkers over time in the different treatment groups and to assess their effect, we used a linear mixed model (LMM). We used results from the first 48 hours of sampling since this is the time window of interest.

Following our hypothesis, LTA release and biomarker response after the start of treatment will not have a linear relation. Therefore, we used polynomial splines to model the trend of LTA release and biomarker response. Changes in biomarkers were assessed by comparing change within the first and second 24 hours after treatment with a T-test. Statistical analyses were performed using SPSS (IBM Software) version 23.

RESULTS

Between January 2013 and May 2014, a total of 41 patients with community-acquired pneumonia were included. After the empirical start of antibiotic treatment in all study patients, 17 of them were found to have pneumococcal pneumonia. Of these 17 patients,13 were in the intervention group, while four were in the control group. In these 13 patients, ten completed the 48 hours (four dosages) of rifampicin treatment, two received three dosages and one received two dosages. The median number of infected lobes was one.

The median age of the total cohort was 69 years, 58% was male, and median CURB-65 score was 2 (**Table 1**). Twenty-six patients received ciprofloxacin as empirical treatment on top of a β-lactam antibiotic with or without rifampicin. Since groups are small, some differences exist between the treatment groups. Baseline characteristics are outlined in **Table 1** (and **Table S1**).

Table 1. Baseline characteristics

	Complete cohort n=41	Rifampicin + β-lactam treatment (*S. pneumoniae*) n=13	β-lactam treatment (*S. pneumoniae*) n=4	P value	Rifampicin + β-lactam treatment (all patients) n=28	β-lactam treatment (all patients) n=13	P value
Medical history							
Median age (IQR)	69 (57-75)	69 (58-76)	48 (42-63)	0.03	71 (61-76)	67 (50-71)	0.13
Female gender	17 (42%)	3 (23%)	4 (100%)	0.01	9 (32%)	8 (62%)	0.08
Cardiovascular disease	11 (27%)	4 (31%)	0 (0%)	0.52	8 (29%)	3 (23%)	0.71
Immunocompromised	12 (29%)	3 (23%)	0 (0%)	0.54	8 (29%)	4 (31%)	0.89
Pulmonary comorbidity	18 (44%)	5 (39%)	1 (25%)	0.62	10 (36%)	8 (62%)	0.12
Influenza vaccination	25 (61%)	7 (54%)	1 (25%)	0.31	16 (57%)	9 (69%)	0.46
Objective parameters at presentation							
Median CURB-65 score (IQR)	2 (1-3)	2 (2-3)	2 (1-2)	0.63	2 (2)	2 (3)	0.68
Pneumonia on chest X ray or confirmed by physical examination	39 (95%)	11 (85%)	4 (100%)	1.00	27 (96%)	13 (100%)	1.00
Causative agent*							
S. pneumoniae	17	13	4	-	13	4	0.34
H. influenza	1	0	0	-	0	1	0.32
S. aureus	1	0	0	-	1	0	0.32
Influenza A	3	1	0	1.00	2	1	1.00
RSV	1	0	0	-	0	1	0.32
Metapneumovirus	2	0	1	0.24	1	1	0.54
Human rhinovirus	5	3	0	0.54	4	1	0.55
Human Coronavirus	1	1	0	1.00	1	0	0.32
Parainfluenza virus 1	2	0	1	0.24	0	2	0.10
Parainfluenza virus 2	1	1	0	1.00	1	0	0.32
No pathogen detected	16	0	0	-	12	4	0.46
Bacterial with viral coinfection	6	4	1	0.83	5	1	0.39
Empirical antibiotic treatment							
Benzylpenicillin/cefuroxime	37/4	12/1	2/2	0.12	27/1	10/3	0.16
Ciprofloxacin/no ciprofloxacin	26/15	7/6	3/1	0.45	16/12	10/3	0.46

IQR, Interquartile range. *in some patients more than one causative agent was detected.

The diagnosis of pneumococcal pneumonia in the 17 patients was based on positive blood cultures in five; positive sputum cultures in six; positive BinaxNOW antigen test in nine; and a positive IMIA test in ten patients. Various *S. pneumoniae* serotypes were detected. Interestingly, two patients had an infection with more than one serotype (**Table S2**).

LTA release and LTA mediated inflammatory response

In short, LTA release could not be demonstrated with two commercial ELISA tests. Of two study patients with proven pneumococcal pneumonia with pneumococcal bacteremia, no LTA mediated inflammatory response via TLR2 was detected.

Results of the laboratory work on LTA response and LTA mediated inflammatory response are described in the supplementary data on the laboratory work.

Clinical outcome is not different between treatment groups

Time to clinical stability and time to defervescence in patients with pneumococcal pneumonia did not differ significantly between treatment groups (**Figure 1A** and **1B**). None of the patients with pneumococcal pneumonia died in the hospital or within 30 days, while 90-day overall mortality was 6%. The median length of hospital stay was four days, and there were no significant differences in ICU admissions, adverse events and recovery at 30 and 90 days in the pneumococcal group and the complete cohort. Clinical outcome parameters are described in **Table 2** and **3**.

Table 2. Clinical outcome parameters for patients with microbiologically proven pneumococcal pneumonia

	All patients n=17	Rifampicin + β-lactam treatment n=13	β-lactam treatment n=4	P value
Median length of hospital stay (IQR)	4 (3-9)	5 (4-9)	4 (2-8)	0.36
ICU admission	4	3	1	0.94
Median length of ICU stay (IQR)	4 (2-6)	3 (2-5)	4	0.66
Mechanical ventilation	1	1	0	0.57
Multiple organ failure	5	4	1	0.83
In hospital mortality	0	0	0	-
Day 30 mortality	0	0	0	-
Day 30 recovery				0.28
Complete	4	2	2	
Partial	10	8	2	
No	3	3	0	
Day 90 mortality	1	1	0	0.57
Day 90 complete recovery	11	8	3	0.53

IQR, interquartile range

Table 3. Clinical outcome parameters for all patients

	Complete cohort n=41	Rifampicin + β-lactam treatment n=28	β-lactam treatment n=13	P value
Median length of hospital stay (IQR)	4 (3-8)	4 (3-8)	4 (2-7)	0.46
ICU admission	7	4	3	0.49
Median length of ICU stay (IQR)	3 (2-7)	5 (2-10)	3 (3-4)	0.59
Mechanical ventilation	2	2	0	0.15
Multiple organ failure	6	4	2	0.21
In hospital mortality	1	1	0	0.49
Day 30 mortality	1	1	0	0.49
Day 30 complete recovery	13	10	3	0.47
Day 90 mortality	2	2	0	0.32
Day 90 complete recovery	25	18	7	0.19

IQR, interquartile range

Biomarker and transcription profiles cannot distinguish treatment groups

The biomarkers CRP, PCT, and MR-proADM were measured at various time points before and after the start of treatment (**Figure 2**). Before the start of the treatment, median CRP and MR-proADM were slightly higher in the rifampicin intervention group, whereas median PCT was slightly higher in the group treated without rifampicin. After the start of treatment, biomarker levels were not significantly different between the groups in the linear mixed model (**Figure 2A-2F and Table 4**).

CRP values showed a small increase within the first 24 hours after the start of treatment in both treatment groups (**Figure 2A, 2D**). In patients with pneumococcal pneumonia, all biomarkers show a steady decline between 24 and 48 hours after the start of treatment (**Figure 2A-2C**). The change in the concentrations of the biomarkers were not different between groups in the first and second 24 hours after the start of treatment (**Table 5** and **Table S3**). In four patients, blood samples (n=5) were limited to those taken during hospitalization.

At inclusion, and 24 hours and 30 days after inclusion, RNA expression profiling of 80 transcripts was performed. The MLPA heat map shows colored quantities of the various transcripts in **Figure 3**. Patients with similar transcript profiles are plotted adjacent. Although nine patients with pneumococcal pneumonia with rifampicin clustered together, the gene expression data do not reveal clear patterns associated with treatment or disease status.

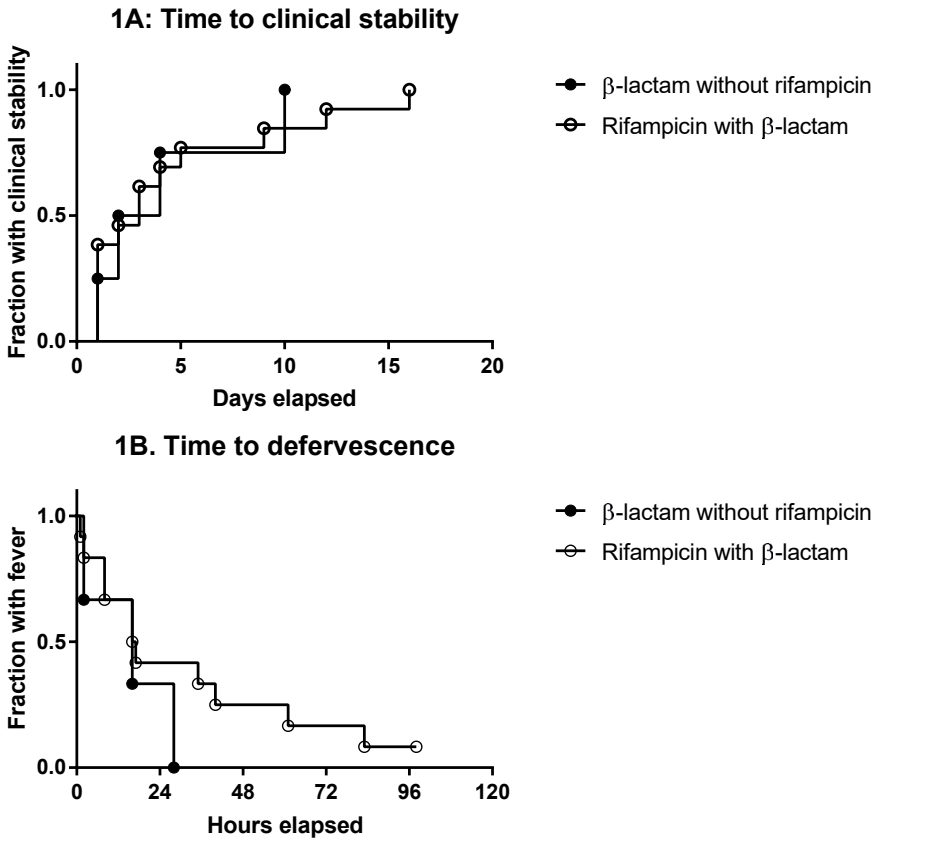

Figure 1. Time to clinical stability and to defervescence in patients with pneumococcal pneumonia.
Kaplan Meier curves for time to clinical stability and time to defervescence in patients with pneumococcal pneumonia treated with rifampicin versus patients treated without rifampicin.

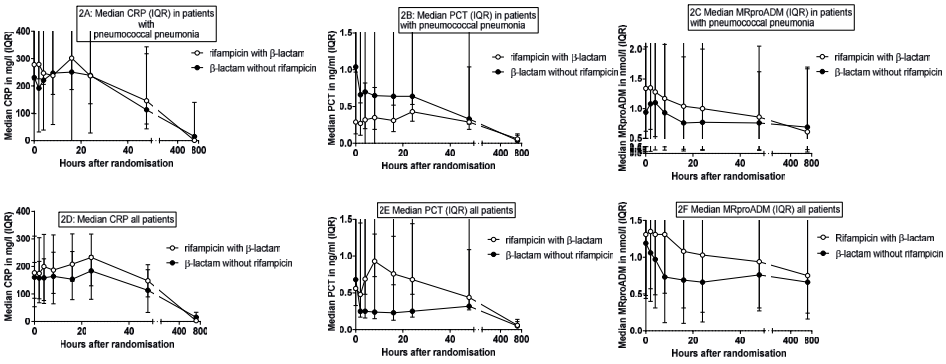

Figure 2. Biomarkers in patients´ plasma before, during and after treatment
The inflammation biomarkers C-reactive protein (CRP), procalcitonin (PCT) and midregional pro-adrenomedullin (MR-proADM) were analysed in plasma. Median biomarker with interquartile range (IQR) over time for patients with pneumococcal pneumonia (2A-2C) and for all patients (2D-2F).

Table 4. Linear mixed model: mean response over time (0-48 hours) in patients with pneumococcal pneumonia treated with rifampicin compared to the control group without rifampicin

Biomarker	Estimate (95% CI)	P value
CRP	37.7 (-32.9 - 108.2)	0.27
PCT	0.00 (-0.07 - 0.07)	0.97
MR-proADM	-0.23 (-0.54 - 0.07)	0.12

CRP, C-reactive protein; PCT, procalcitonin; MR-proADM, midregional pro-adrenomedullin. The group without rifampicin is the baseline comparator.

Table 5. Change in biomarkers over time in patients with pneumococcal pneumonia

	Rifampicin group (n=13)	Group without rifampicin (n=4)	Mean difference (95% CI)	P value*
In the first 24 hours after start of treatment				
ΔCRP	13.7 mg/L	32.8 mg/L	-19.0 (-113.6-75.5)	0.67
ΔPCT	3.70 ng/mL	0.03 ng/mL	3.67 (-12vw.18-19.52)	0.63
ΔMR-proADM	-0.20 nmol/L	-0.21 nmol/L	-0.00 (-0.34-0.35)	0.98
In the second 24 hours after start of treatment				
ΔCRP	-79.3 mg/L	-112.6 mg/L	33.3 (-51.4-117.9)	0.42
ΔPCT	-1.89 ng/mL	-0.29 ng/mL	-1.60 (-6.19-2.99)	0.47
ΔMR-proADM	-0.28 nmol/L	-0.13 nmol/L	-0.15 (-0.69-0.39)	0.57

CRP, C-reactive protein; PCT, procalcitonin; MR-proADM, midregional pro-adrenomedullin. * T-test
Change in concentrations of CRP, PCT and MR-proADM within the intervention (rifampicin) group and within the control group (without rifampicin) in the first 24 hours after start of treatment, i.e. from start of treatment until 24 hours thereafter; and in the second 24 hours after start of treatment, i.e. from 24 to 48 hours after start of treatment. Mean difference between intervention and control group and the P value are shown in separate columns.

To identify transcripts with the highest discriminatory power between pneumococcal versus other infections PLS-DA were run and VIP scores were calculated. Transcripts with the five highest VIP scores are shown in **Figure S1A-E**. Only Chemokine (C-C motif) ligand 5 (*CCL5*) was statistically significant lower at 24 hours after the start of treatment in patients with pneumococcal pneumonia versus patients with non-pneumococcal pneumonia. Treatment with or without rifampicin did not significantly affect the results.

DISCUSSION

The PRISTINE study is the first exploratory clinical trial in humans to determine the feasibility of adding rifampicin to the standard treatment with β-lactams of community-acquired pneumococcal pneumonia. The rifampicin is added to reduce the release of bacterial compounds within the first hours of therapy and thereby attenuate the inflammatory re-

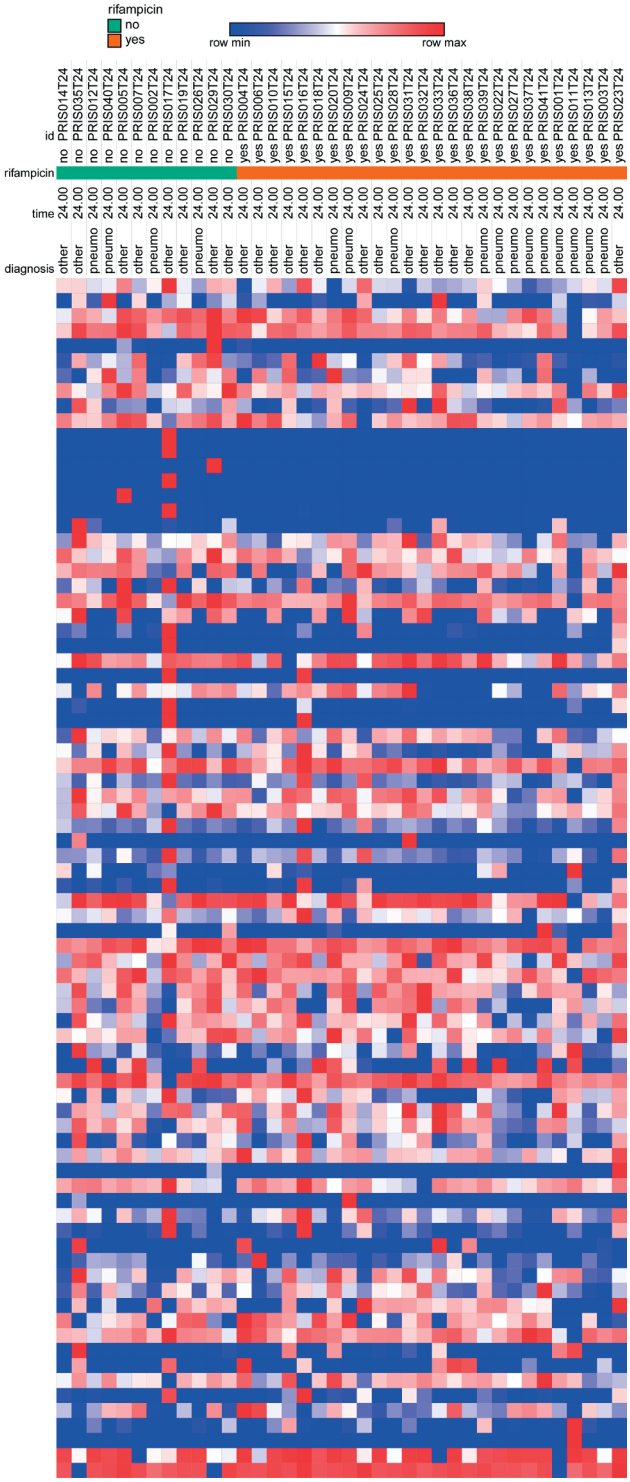

Figure 3. Heatmap of RNA expression results measured by MLPA in all patients

Heatmap at T=24 hours with rifampicin treated patients (brown) versus patients treated without rifampicin (green). Clustering is poor for all genes investigated irrespective of clinical diagnosis.

sponse. In this initial small group, the additional non-lytic rifampicin antibiotic versus lytic β-lactam antibiotic only treatment for pneumococcal pneumonia did not reveal differences in the blood concentrations of various inflammatory biomarkers nor in the clinical response to treatment.

Strengths of our study are the high percentage of pneumococcal infections included, the frequent sequential measurement of a spectrum of biomarkers in the first 48 hours to assess our hypothesis, and the complete biomarker profile to evaluate the specific inflammatory responses. Initially, we included only patients with high severity score (CURB-65≥2) as the percentage of pneumococcal infection is highest in this group, and the high severity would best contrast the possible effects. After inclusion of the eighth study patient, we extended our inclusion criteria to patients having a specific risk factor for pneumococcal pneumonia to speed up inclusions.[17] We applied extensive testing for pneumococcal infection, to ensure the identification of all patients with pneumococcal pneumonia.[23] We were able to confirm a pneumococcal infection in 41% of patients. This percentage is higher than in comparable hospital and intensive care studies with community-acquired pneumonia.[11,26,27]

In vitro studies and animal models demonstrated differences in LTA release and inflammatory response within hours in lytic versus non-lytic antibiotic treatment of *S. pneumoniae*.[12,28,29] Although extensive sampling is a challenge in human trials, it is essential for testing our hypothesis. Therefore, the large number of sequential samples we collected is an important strength of our study. With the extensive sampling, we detected that CCL5 is expressed significantly differently between pneumococcal pneumonia versus non-pneumococcal pneumonia 24 hours after start of treatment. CCL5 is known to be upregulated in pneumococcal infection and to be an essential chemokine in pneumococcal adaptive immunity.[30] Our finding needs to be validated in a larger cohort of pneumonia patients.

A weakness of our pilot trial is the small sample size. This is however in line with the exploratory character of our study. As we anticipated that the LTA and biomarker responses induced by β-lactam treatment would be in a broad range, we included more patients with rifampicin added to β-lactam treatment than β-lactam treatment only, and randomized at a 2:1 ratio. With only four patients with pneumococcal pneumonia treated with β-lactam therapy only, this assumption was imperfect and the small group hindered comparisons. For example, in the analyses of biomarkers for inflammation, at start of treatment, PCT value seems higher in the β-lactam group while CRP and MR-proADM show higher values in the rifampicin group. Since only three samples (one sample was missing) were available in the β-lactam group, the interpretation of these findings is difficult.

We could not detect LTA in plasma nor its direct inflammatory response via TLR2. LTA cell wall components should bind TLR2 and induce the release of a broad range of proinflammatory cytokines leading to neutrophil-mediated lung damage and, with that, morbidity and mortality.[31,32] Most likely, an inhibiting effect of human plasma contributes to the low immune response in these patients. In addition, with a median number of only one infected lung lobe, representing relatively limited pneumococcal load, LTA plasma concentration could be too low to mount a response via TLR2 *in vitro* (see supplementary data), but may nonetheless have an effect *in vivo.*

LTA release may also have been delayed by quinolone treatment.[14,29] Ciprofloxacin was frequently co-administered in our cohort. A delayed LTA release may have decreased the potential difference in inflammatory responses between the two treatment groups.

Finally, another reason for the absence of detectable LTA in our samples could be the serotypes causing pneumococcal pneumonia. Different pneumococcal isolates have different lytic effects.[33] In an experimental meningitis model in rabbits, serotype 23F caused more LTA release and inflammation than pneumococcal serotype 3.[34] In our study, only one patient had a pneumococcal pneumonia with serotype 23F versus four patients with serotype 3.

In contrast to LTA in plasma, LTA can be detected at the site of infection in humans (see supplementary data). For example, in liquor of patients with pneumococcal meningitis, LTA is detectable until 15 days after the start of treatment.[35] Unfortunately, it is not possible to puncture the infected lung lobe for repeated measurements in critically ill human patients. Therefore, human studies to determine the LTA load in the lung during pneumonia have not been done.

Previous *in vitro* and animal studies showed vast differences in LTA release and inflammatory response between lytic versus non-lytic antibiotic treatment. The potential clinical benefit of decreased LTA release and inflammatory response in patients with pneumococcal pneumonia might be substantial. Restrepo *et al.* demonstrated that patients with community-acquired pneumonia who were transferred to the ICU immediately from the emergency department were better off than patients who were initially treated on wards and thereafter transferred to ICU.[36] This secondary deterioration could be caused by inflammation due to LTA release after the start of treatment.

A large randomized trial of patients with Gram-positive *Staphylococcus aureus* bacteraemia showed no adjunctive clinical benefit of rifampicin over standard (most often

flucloxacillin) antibiotic treatment.[37] Long-term endpoints in that trial were used, making a comparison with our short-term outcome measures difficult.

Strategies to dampen inflammatory response in pneumonia have so far primarily focused on corticosteroids. Corticosteroid therapy demonstrated shorter time to clinical stability and limited shortening of hospital stay in patients with non-severe community-acquired pneumonia. Some studies in adults with severe disease, show a reduction in mortality. The quality of these studies is moderate. In all studies, corticosteroid therapy increased the risk of hyperglycemia.[38] Therefore, corticosteroids are not included in current treatment guidelines.[7,8]

Alternative therapeutic options should be explored to attenuate the inflammation.

The effects and benefits of non-lytic antibiotics for the treatment of pneumococcal infections may be easier to detect and prove in pneumococcal meningitis patients. In this group of patients with high morbidity, long-term sequelae, and substantial mortality strategies to improve outcomes are urgently needed.[39] Moreover, the clinical results of our study could have been blurred by the use of antipyretics.

Higher LTA concentration in liquor in human patients with pneumococcal meningitis is associated with worse outcome.[40] In addition, in rabbits with pneumococcal meningitis, rifampicin reduced LTA release and inflammatory response, and improved survival substantially.[13] Therefore, clinical trials with non-lytic antibiotics in pneumococcal meningitis should be developed. Rifampicin would be the antibiotic of choice since it is most effective in killing *S. pneumoniae* while causing the least release of LTA per killed bacterial cell.[41]

Unfortunately, we could not compare monotherapy of a non-lytic (rifampicin) antibiotic versus monotherapy of a lytic, β-lactam, antibiotic. This would be a highly relevant, but different research question. Reasons for this are that the current Dutch guidelines for community-acquired pneumonia recommend β-lactam antibiotic (e.g., benzylpenicillin) treatment and the fact that rifampicin monotherapy may induce resistance during treatment. Therefore, it would have been unethical to withhold this first-line treatment to patients with community acquired pneumonia. A significant difference in LTA release has been demonstrated in a rabbit model of *S. pneumoniae* meningitis, when comparing β-lactam monotherapy with rifampicin followed by β-lactam antibiotic therapy six hours later.[42] In the rifampicin treatment group in our study, rifampicin was frequently (56%) given before β-lactam treatment, but with a median time frame of 5 minutes only (interquartile range –10 minutes to 60 minutes). Therefore, the antimicrobial killing of *S. pneumonia* in both groups might be primarily caused by the β-lactam (lytic) killing effect.

In conclusion, the PRISTINE exploratory study demonstrated the feasibility of adding rifampicin to β-lactam antibiotics in the treatment of community-acquired pneumococcal pneumonia but, despite solid *in vitro* and experimental animal research evidence, failed to demonstrate a difference in LTA and subsequent inflammatory response. Further studies in selected groups of patients, such as those with pneumococcal meningitis, will be necessary to confirm the hypothesis that non-lytic antibiotic treatment attenuates inflammatory response and improves clinical outcome.

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SUPPLEMENTARY DATA

- Part 1: Additional tables and figure (Table S1-S3 and Figure S1)
- Part 2: Laboratory work PRISTINE

Table S1. Additional baseline characteristics

	Complete cohort n=41	Rifampicin + betalactam treatment (pneumococcal pneumonia) n=13	Betalactam treatment (pneumococcal pneumonia) n=4	P value	Rifampicin + betalactam treatment (all patients) n=28	Beta-lactam treatment (all patients) n=13	P value
Medical history							
Hospital admission in the previous year	13 (32%)	4 (31%)	0 (0%)	0.52	10 (36%)	3 (23%)	0.42
Antibiotic use in the previous 3 months	17 (42%)	4 (31%)	3 (75%)	0.12	10 (36%)	7 (54%)	0.27
Help needed with activities of daily living	3 (7%)	1 (7%)	0 (0%)	1.00	2 (7%)	1 (8%)	1.00
Current smoker	11 (27%)	5 (38%)	2 (50%)	0.68	7 (25%)	4 (31%)	0.70
Smoking history	34 (83%)	12 (92%)	3 (75%)	0.43	23 (82%)	11 (84%)	0.85
Median number of pack years (IQR)	20 (6-45)	20 (9-43)	31 (4-50)	1.00	21 (7-49)	20 (4-43)	0.75
Travelled abroad in previous 3 months	12 (29%)	2 (15%)	1 (25%)	1.00	8 (29%)	4 (31%)	0.89
Symptoms at presentation							
Symptoms < 1 week	35 (85%)	11 (85%)	3 (75%)	1.00	24 (86%)	11 (85%)	0.93
Acute onset of symptoms	15 (37%)	6 (46%)	0 (0%)	0.09	11 (39%)	4 (31%)	0.60
Throat pain	12 (29%)	3 (23%)	2 (50%)	0.30	7 (25%)	5 (38%)	0.38
Runny nose	16 (39%)	4 (31%)	3 (75%)	0.12	9 (32%)	7 (54%)	0.19
Cough	35 (85%)	13 (100%)	4 (100%)	-	25 (89%)	10 (77%)	0.30
Sputum production	26 (63%)	10 (77%)	3 (75%)	1.00	17 (61%	9 (69%)	0.60
Dyspnea	33 (80%)	11 (85%)	4 (100%)	1.00	22 (79%)	11 (85%)	0.65
Pleuritic chest pain	16 (39%)	7 (54%)	2 (50%)	0.89	12 (43%)	4 (31%)	0.46
Fever	37 (90%)	12 (92%)	3 (100%)	0.43	26 (93%)	11 (85%)	0.41
Myalgia	12 (29%)	5 (38%)	2 (50%)	0.68	7 (26%)	5 (39%)	0.42
Headache	17 (42%)	8 (62%)	3 (75%)	0.62	13 (46%)	4 (31%)	0.34
Joint pain	10 (24%)	3 (23%)	1 (25%)	0.94	8 (29%)	2 (15%)	0.36
Objective parameters at presentation							
Leukocyte count > 15 x10e9/l	14 (34%)	6 (46%)	2 (50%)	0.89	9 (32%)	5 (39%)	0.69
On chest X-ray: an alveolar pattern (lobar, segmental or sub-segmental infiltrate)	25 (61%)	8 (62%)	4 (100%)	0.14	18 (64%)	7 (54%)	0.52
Median number of lobes infected (IQR)	1 (1-2)	1 (1-2)	1 (1-2.5)	0.47	1 (1-2)	1 (1-1.5)	0.56

Table S2. Pneumococcal serotypes

Pneumococcal serotype	Number of cases
1	2*
3	4*
8	3
11A	1
18C	1*
20	1
23F	1*

* One patient with two serotypes; one patient with three serotypes detected.
These findings are ambiguous and could be caused by infection with multiple pneumococcal serotypes, asymptomatic carriage or previous infection with other serotypes than the one causing the actual infection or false positive test result.

Table S3. Difference in biomarkers in complete cohort (n=41)

Biomarker per time frame	Rifampicin group (n=28)	Group without rifampicin (n=13)	Mean difference (95% CI)	P value*
In the first 24 hours				
CRP	16.8 mg/L	41.8 mg/L	-25.0 (-84.3 - 34.2)	0.40
PCT	2.0 ng/mL	1.4 ng/mL	0.65 (-5.4 - 6.7)	0.83
MR-proADM	-0.08 nmol/L	-0.07 nmol/L	-0.01 (-0.25 - 0.22)	0.90
In the second 24 hours				
CRP	-65.0 mg/L	-50.5 mg/L	-14.5 (-61.0 - 32.0)	0.53
PCT	-1.21 ng/mL	-0.82 ng/mL	-0.40 (-2.44 - 1.65)	0.70
MR-proADM	-0.26 nmol/L	-0.05 nmol/L	-0.21 (-0.50 - 0.07)	0.14

* T-test

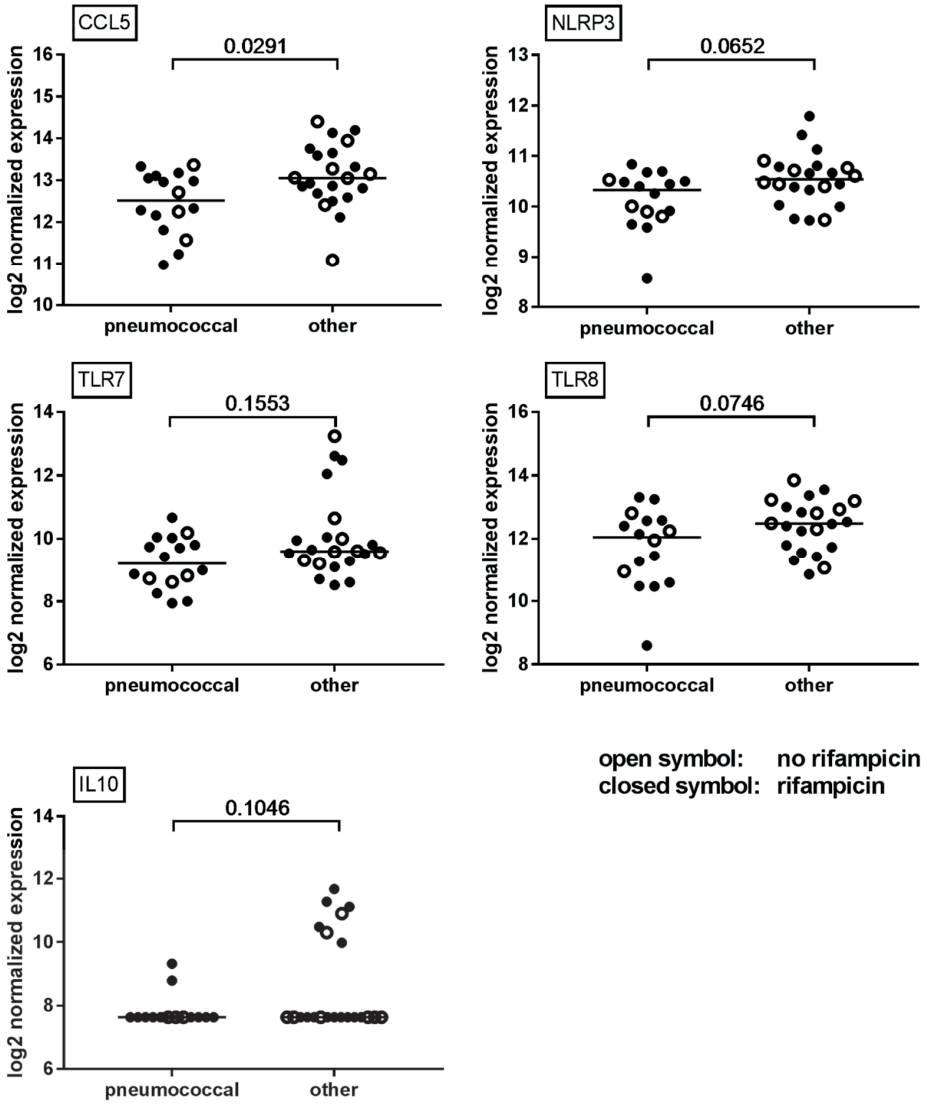

Figure S1. Transcripts with the top 5 VIP scores in the first 24 hours

Transcripts with the five highest VIP scores after 24 hours of treatment to distinguish between pneumococcal pneumonia and non-pneumococcal pneumonia (other). *CCL5* is the only transcript that is significantly different between pneumococcal infection and non-pneumococcal infection 24 hours after the start of treatment.

Laboratory work PRISTINE

During inclusion of study patients in the PRISTINE study, laboratory tests were conducted to detect lipoteichoic acid (LTA) in serum and to test TLR2 transfected Human Embryonic Kidney (HEK) 293 cells (Invivogen®, the Netherlands, catalogue number 293-htlr2cd14) for the ability to produce IL-8 after trigger by pneumococcal cell wall components (i.e. LTA), as measure of inflammatory potential. In humans, lipoteichoic acid (LTA) is recognized by Toll-like receptor 2 (TLR2), the pattern recognition receptor on macrophages. Binding of LTA to TLR2 induces the release of proinflammatory cytokines (e.g. IL-1, IL-6, TNF) and neutrophil influx.[1,2]

To measure LTA we used two commercial LTA ELISA kits (SunRedBio, China, catalogue number 201-12-1911 and EIAab Science Co LTD, China, catalogue number E1405Ge).

Firstly, *Staphylococcus aureus* and *Streptococcus pyogenes* LTA (Sigma-Aldrich, Zwijndrecht, the Netherlands, L2515 and L3140) solution was made with pure water. In various concentrations (0.31-20 ng/mL), the LTA levels remained under the detection limit (0.3 ng/mL) of the ELISA.

Secondly, we cultured *Streptococcus pneumoniae* in vitro. Neither in brain heart infusion growth medium (BHI solution) with *S. pneumoniae*, nor in BHI solution with *S. pneumoniae* and various concentrations of benzylpenicillin nor in BHI with *S. pneumoniae* and various concentrations of rifampicin, significant amounts of LTA were detected with ELISA (Table S4).

In conclusion, both ELISA tests were unable to demonstrate LTA in various concentrations in water and were unable to demonstrate any LTA released from the cell wall in pneumococcal broths with and without two types of antibiotics (lytic and non-lytic).

Thereafter, we determined TLR2 responsiveness with TLR2 transfected Human Embryonic Kidney (HEK) 293 cells (Invivogen®, the Netherlands, 293-htlr2cd14). TLR2 transfected HEK 293 cells were used to measure IL-8 production by ELISA (Invitrogen®, the Netherlands, CHC1303) in response to TLR2 stimulation.[3] Positive control for the HEK293 cells was Pam3Cys-SKKKK (EMC microcollections®, Germany, L2000) and negative control with ultrapure lipopolysaccharide (LPS) (Invivogen®, the Netherlands, tlrl-peklps) was added. IL-8 release was measured quantitatively with ELISA. Higher IL-8 release represents higher TLR2 binding by immunoreactive agents.

In our experiment, *S. aureus* LTA showed higher IL-8 response with stimulation in increasing LTA concentrations (Figure S2).

Table S4. LTA ELISA response in *S. pneumoniae* log culture with and without antibiotics

	Concentration	Time	concentration LTA
	(µg/mL)	(minutes)	(ng/mL)
Without antibiotic		10	0.32
Rifampicin	10	10	0.23
		30	0.32
		60	0.23
		90	0.21
Rifampicin	1	10	0.26
		30	0.35
		60	0.25
		90	0.61
Penicillin	1	10	0.21
		30	0.25
		60	0.22
		90	0.23
Penicillin	0.1	10	nt
		30	0.43
		60	nt
		90	0.32

All *S. pneumoniae* log cultures showed significant and comparable reduction in colony forming units after the start of treatment with penicillin and rifampicin.
nt = not tested
EIAab Science Co LTD, China, catalogue number E1405Ge

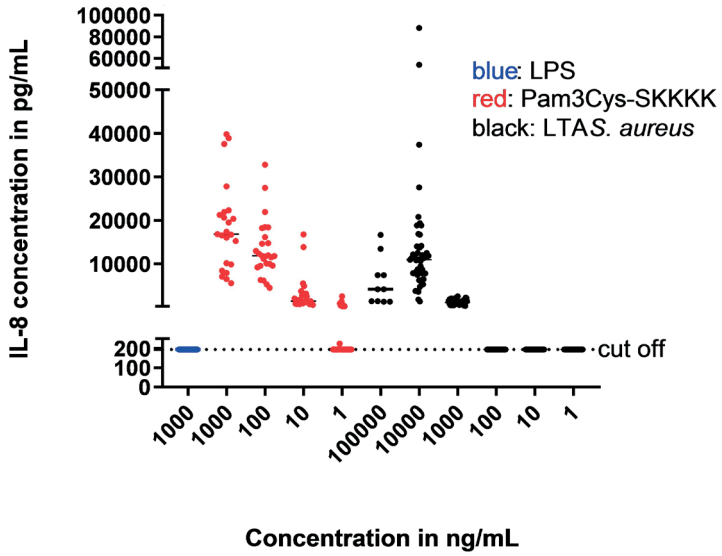

Figure S2. *S. aureus* LTA induced IL-8 response

Upon adding human serum to the *S. aureus* LTA samples, the IL-8 response was reduced. When serum was added to the positive control, Pam3Cys-SKKKK, the IL-8 response was much less reduced. This implied an LTA-inhibiting component in human serum. This inhibiting factor does not affect the functionality of the TLR2 receptor since IL-8 response to a positive control was less reduced (Figure S3).

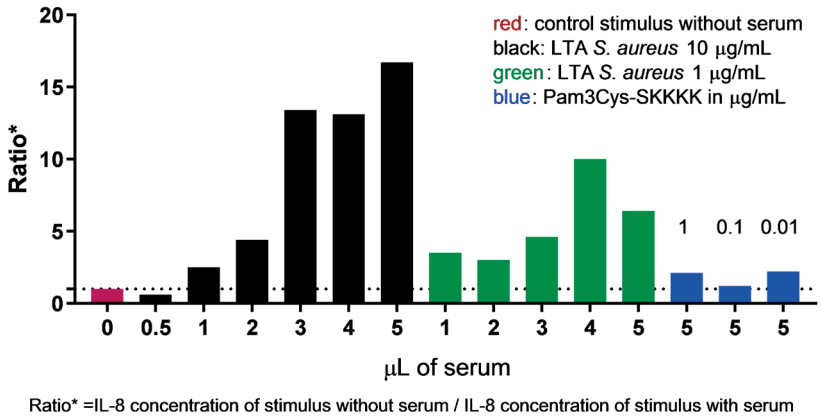

Figure S3. Inhibition of TLR2 response with human serum

The *S. pneumoniae* log culture samples did elicit a TLR2 response. The response in the BHI solution of *S. pneumoniae* with benzylpenicillin was stronger than the response in the solution with rifampicin (Figure S4).

Of two patients from the PRISTINE study with proven pneumococcal pneumonia with pneumococcal bacteremia, plasma was thawed and 5 µL was incubated with 5x10e4 HEK293 cells. Positive control for the HEK293 cells was Pam3Cys-SKKKK, positive control for the LTA was *S. aureus* LTA and negative control with ultrapure LPS was added. IL-8 release was measured quantitatively with ELISA.

No TLR2 response could be detected after adding EDTA plasma to cell cultures. Also after diluting the samples (1:10, 1:25 and 1:50), to remove a potentially inhibiting effect of plasma, no TLR2 response was detected. Similarly, on testing a few urine samples of this subset, in none of them a TLR2 response could be detected.

Of two patients with pneumococcal meningitis and in a third with pneumococcal empyema, a strong TLR2 mediated inflammatory response was measured with cerebrospinal fluid and pleural fluid respectively. These samples were not collected in the PRISTINE study (Figure S5).

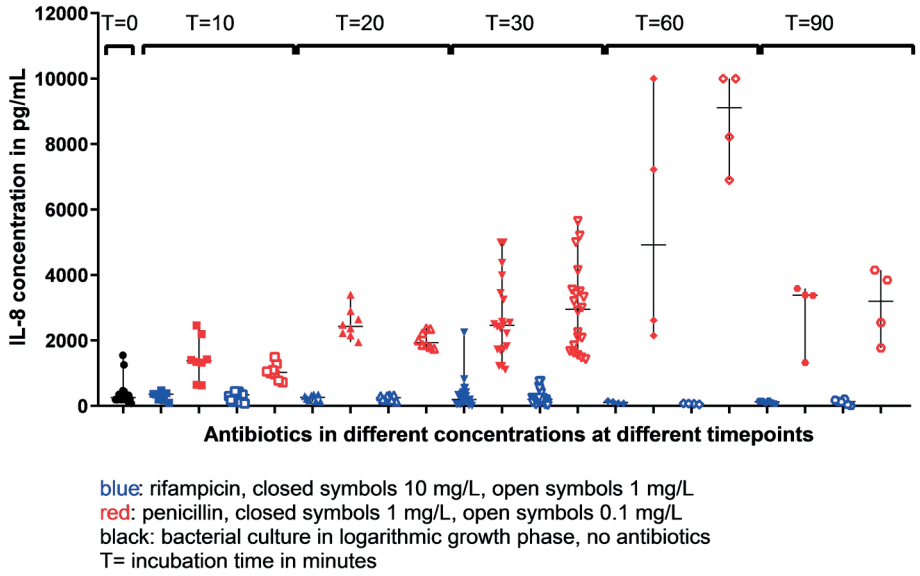

Figure S4. TLR2 response of *S. pneumoniae* with and without antibiotics

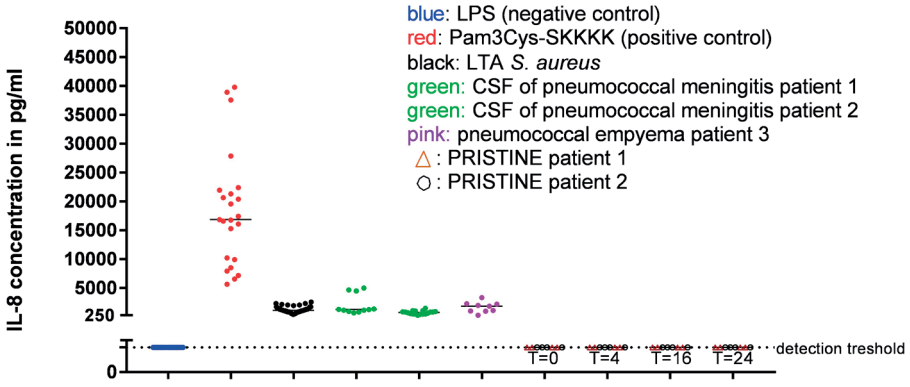

Figure S5. TLR2 mediated inflammatory response in clinical samples
CSF: cerebrospinal fluid

Conclusion

TLR2 transfected HEK 293 cells are able to respond to LTA in *in vitro* samples and in cerebrospinal fluid and pleural fluid, but not in patient plasma samples from the PRISTINE study. Most likely, an inhibiting effect of human serum might contribute to the low immune response in these experiments.

In addition, plasma concentration in patients with pneumococcal pneumonia might be too low to mount an IL-8 response in vitro.

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