Establishing the lowest penicillin concentration to prevent pharyngitis due to *Streptococcus pyogenes* using a human challenge model (CHIPS): a randomised, double-blind, placebo-controlled trial

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Summary

Background The in-vivo plasma concentration of penicillin needed to prevent *Streptococcus pyogenes* pharyngitis, recurrent acute rheumatic fever, and progressive rheumatic heart disease is not known. We used a human challenge model to assess the minimum penicillin concentration required to prevent streptococcal pharyngitis.

Methods In CHIPS, a randomised, double-blind, placebo-controlled, human challenge trial, healthy adult volunteers were randomly assigned by a computer-generated random sequence to target steady-state penicillin plasma concentrations (placebo, 3, 6, 9, 12, or 20 ng/mL). The study was a single-centre trial held in Perth, WA, Australia. Participants had to be healthy adults, aged 18–40 years, at low risk of complicated *S pyogenes* disease, and without high type-specific IgG antibodies against the *emm*75 *S pyogenes* challenge strain. Participants and staff involved in clinical care remained masked to treatment allocation for the duration of the study. Individualised 5-day continuous intravenous infusions of penicillin were commenced 12 h before direct pharyngeal application of the *emm*75 challenge strain. The primary endpoint was clinical pharyngitis. This trial is registered on the Australian New Zealand Clinical Trials Registry, ACTRN12621000751875, and is completed.

Findings Between Aug 23, 2022, and July 31, 2023, 60 participants were randomly assigned (35 [58%] were female and 25 [42%] were male), with 57 included in the analysis. The clinical pharyngitis endpoint was met in eight (57%) of 14 in the placebo group, four (44%) of nine in the 3 ng/mL target steady-state penicillin plasma concentration group, four (44%) of nine in the 6 ng/mL group, none of eight in the 9 ng/mL group, none of eight in the 12 ng/mL group, and none of nine in the 20 ng/mL group. No severe or serious adverse events occurred. Using Bayesian concentration–response modelling, the minimum steady-state plasma concentration of penicillin for which 90% of participants would avoid clinical pharyngitis was 8·1 ng/mL (95% credible interval 6·1–10·9).

Interpretation When steady-state penicillin concentrations are greater than 9 ng/mL, few people will develop experimental *emm*75 *S pyogenes* pharyngitis. These data will inform efforts to improve long-acting penicillin preparations and dosage regimens to prevent recurrent rheumatic fever and rheumatic heart disease.

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Introduction

Rheumatic fever and rheumatic heart disease are complications of untreated *Streptococcus pyogenes* infections of the throat and possibly the skin.[1] Globally, there are more than 40 million people living with rheumatic heart disease.[2] Secondary prophylaxis, delivered as intramuscular benzathine benzylpenicillin in 900 mg doses (1 200 000 units) every 3–4 weeks, remains the only proven intervention to prevent further infections with *S pyogenes*, recurrent rheumatic fever, and progressive rheumatic heart disease.[1–4]

Use of benzathine benzylpenicillin for secondary prophylaxis has changed very little since its efficacy was first demonstrated in the 1950s.[4] Following injection, benzathine benzylpenicillin slowly dissolves and dissociates to benzylpenicillin, which is absorbed into the bloodstream.[5,6] Traditionally, a plasma penicillin concentration of 0·02 µg/mL (20 ng/mL) has been accepted as the target for prevention of *S pyogenes* pharyngitis, based on the in-vitro minimum inhibitory concentrations (MICs) for most strains of *S pyogenes*.[7,8]

Contemporary pharmacokinetic studies have shown that most patients do not sustain plasma benzylpenicillin concentrations above 20 ng/mL across the entire dosage interval between benzathine benzylpenicillin injections.[9,10] However, adequate adherence to planned injections does protect against breakthrough rheumatic fever episodes,

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Research in context

Evidence before this study

Use of benzathine benzylpenicillin for secondary prophylaxis of acute rheumatic fever and rheumatic heart disease has changed very little since its efficacy was first demonstrated in the 1950s. We completed a PubMed search for *Streptococcus pyogenes* human infection studies published before Jan 1, 2021, with no language restrictions, using combinations of the search terms "streptococcus pyogenes", "group A streptococcus", "penicillin", "experimental", "human infection", "human challenge", "rheumatic heart disease", and "randomised control trial". There were four *S pyogenes* human infection studies reporting a total of 195 participants, three of which were placebo-controlled vaccine trials conducted in the 1970s. We did not identify a human infection study that tested the minimum penicillin concentration required to prevent streptococcal infection. A plasma penicillin concentration of 20 ng/mL (0·02 mg/L) has been historically accepted as a pharmacological surrogate of efficacy for monthly intramuscular injections of benzathine benzylpenicillin to prevent *S pyogenes* pharyngitis and subsequent acute rheumatic fever and rheumatic heart disease. Pharmacokinetic studies show this target is not achieved across the entire dosage interval between injections. However, successful adherence to secondary prophylaxis protects against acute rheumatic fever episodes, suggesting that concentrations lower than 20 ng/mL might be adequate to prevent most *S pyogenes* infections.

Added value of this study

In the 70-year history of benzathine benzylpenicillin as the foundation of secondary prophylaxis for acute rheumatic fever, these data are the first randomised experimental human data defining the minimum penicillin required to avoid streptococcal pharyngitis. No participants who attained a mean steady-state plasma concentration of 9 ng/mL or greater developed pharyngitis, suggesting a protective threshold lower than the historical 20 ng/mL target, and even lower than the in-vitro minimum inhibitory concentration for this well characterised strain (12 ng/mL).

Evidence of a protective threshold concentration lower than the historical target of 20 ng/mL will underpin efforts to improve the delivery and formulation of current long-acting penicillin preparations. Subcutaneous delivery of benzathine benzylpenicillin rather than traditional intramuscular administration might be less painful and increase the time between injections. In addition, these data will inform the target product profile for new formulations, which have the potential to improve injectability and global access for this essential medicine.

Implications of all the available evidence

Development of a less painful and longer lasting injectable secondary prophylaxis regimen is a high priority for consumers and stakeholders. Combined with emerging evidence for less painful, subcutaneous penicillin administration, which enables longer intervals between injections, a lower target plasma penicillin concentration will inform development of a better long-acting penicillin regimen to prevent acute rheumatic fever, rheumatic heart disease, and other streptococcal infections.

suggesting that concentrations lower than 20 ng/mL might be adequate to prevent most *S pyogenes* infections.[9–14] Previous research has shown that meaningful antibacterial effects occur at penicillin concentrations that are below the in-vitro MIC, providing a plausible mechanistic explanation for a lower target.[15,16]

The overall effectiveness of rheumatic fever and rheumatic heart disease prevention programmes is limited by poor adherence to the painful injections, which are recommended for a minimum of 5 years and sometimes must be taken throughout the life course.[17,18] Development of a less painful and longer lasting injectable secondary prophylaxis regimen is a high priority for consumers and stakeholders.[19] The lack of a human in-vivo target protective concentration has hampered efforts to design improved long-acting penicillin formulations. Using an established human challenge model, we aimed to assess the minimum steady-state plasma penicillin concentration required to prevent streptococcal pharyngitis.[20]

Methods

See Online for appendix

Study design

The CHIPS trial was a randomised, double-blind, placebo-controlled, parallel-group, human challenge trial. The single-centre trial took place in an inpatient clinical trials facility (Linear Clinical Research, Perth, WA, Australia). The study protocol, establishment of an *S pyogenes* human challenge model, challenge strain selection, and manufacture have previously been described in detail.[20–22] The study was approved by Bellberry Human Research Ethics Committee, Australia (2021-03-295). The trial was registered on the Australian New Zealand Clinical Trials Registry (ACTRN12621000751875).

Participants

The trial was conducted with healthy adult volunteers aged 18–40 years at low risk of complicated *S pyogenes* disease, and without high type-specific IgG antibodies against the *emm*75 *S pyogenes* challenge strain (in-vitro penicillin MIC of 12 ng/mL).[22] Sex was self-reported with the options of male or female. Recruitment was conducted by Linear Clinical Research, in Perth (WA, Australia). Extensive participant screening included a transthoracic echocardiogram to exclude subclinical cardiac pathology. A full list of inclusion and exclusion criteria is presented in the appendix (p 8).[21] Written informed consent was obtained from all participants. A committee including an independent chair, infectious disease expert, and biostatistician reviewed

safety data and pre-planned interim analyses between each consecutive cohort of 15 participants. Participant remuneration was approved by the ethics committee in recognition of the contribution of time or any inconvenience experienced as a result of participation in research.

Randomisation and masking

Participants were enrolled consecutively into four cohorts of 15 participants each. Using a computer-generated random sequence, participants were assigned to placebo or 3 ng/mL, 6 ng/mL, 9 ng/mL, 12 ng/mL, or 20 ng/mL target steady-state penicillin plasma concentrations. As specified in the protocol,[21] the first three cohorts (45 participants) were randomly assigned 1:1:1:1:1 in blocks of five to five possible allocations (placebo, 3 ng/mL, 6 ng/mL, 12 ng/mL, or 20 ng/mL). Following a planned interim analysis, the 15 participants in the final cohort were randomly assigned 2:3 in blocks of five to placebo and 9 ng/mL, with the aim of increasing precision around the estimate of the protective concentration (appendix p 32).

Participants and staff involved in clinical care remained masked to treatment allocation for the duration of the study, until the statistical analysis plan was finalised and the database closed (appendix p 12). To maintain masking, infusion bags were prepared by an independent compounding pharmacist and transported to the inpatient facility with blinded labelling.[21]

Procedures

Every participant had a pharmacokinetic assessment after a single dose of intravenous benzylpenicillin 7–35 days before random assignment.[21] Based on the resulting estimates of plasma benzylpenicillin volume of distribution and clearance, the required continuous infusion doses to attain any of the possible allocated steady-state concentrations were calculated for each participant. The stability of the buffered benzylpenicillin preparations under simulated trial conditions has previously been demonstrated.[23]

After admission to an inpatient clinical trials unit (day –1), each participant had a midline intravenous catheter inserted. On the following day (day 1), at least 12 h into the continuous infusion, participants were challenged by a standardised procedure involving application of the *emm*75 *S pyogenes* to the pharynx using a sterile Dacron swab, from single-dose vials containing $1-3\times10^5$ bacterial colony forming units in 1 mL animal-free liquid medium (manufactured at Murdoch Children's Research Institute [Melbourne, VIC, Australia] following principles of Good Manufacturing Practice and strict quality control).[24] Steady-state plasma penicillin concentrations were measured every 12 h after starting the infusion using a validated liquid chromatography-mass spectrometry assay.[25] Participants were kept as inpatients and closely monitored for development of symptomatic pharyngitis and adverse events for up to 5 days. Those meeting the primary endpoint within this timeframe were treated with oral azithromycin (500 mg daily for 5 days) at the time of diagnosis, whereas those who did not meet the primary endpoint received the same dosage of oral azithromycin at the end of the observation period on day 5. Participants were discharged approximately 24 h after starting antibiotics and returned for outpatient visits approximately 1 week and 1 month after discharge.

Outcomes

The primary outcome was development of symptomatic *S pyogenes* pharyngitis, assessed according to a predefined clinical case definition (appendix p 11) and point-of-care molecular test (ID NOW STREP A2, Abbott, Scarborough, ME, USA).[21,24] All outcome assessors were medical doctors who received training on the study pharyngitis clinical case definition. Bedside molecular test results were confirmed by standard bacterial culture (PathWest Laboratories, WA, Australia). The secondary outcome was pharyngeal colonisation, defined as detection of *S pyogenes* on 2 consecutive days by quantitative PCR (cycle threshold <30), irrespective of signs and symptoms of pharyngitis. Participants meeting the primary endpoint were considered to have also met the secondary colonisation endpoint. Results of the second secondary endpoint defined in our protocol—identification of target salivary penicillin concentration required to prevent *S pyogenes* pharyngitis or colonisation—will not be reported since it was not technically feasible to assess.

Key exploratory endpoints included C-reactive protein of more than 20 mg/L at any post-challenge timepoint and substantial antibody responses (>0·2 $\log_{10}$ titre increase between pre-challenge and 1-month follow-up) to six streptococcal antigens (deoxyribonuclease B, streptolysin O, group A carbohydrate, streptococcal C5a peptidase, *S pyogenes* cell envelope proteinase, and *S pyogenes* adhesion and division protein) measured by multiplexed electrochemiluminescence assay (Meso Scale Discovery platform, Rockville, MD, USA).[26] A full list of all prespecified endpoints was reported in our protocol.[21]

Safety events were elicited from regular review by study staff during the inpatient stay and outpatient visits, along with documented physical examination findings, clinically significant laboratory results, or other documents relevant to participant safety. All adverse events, adverse reactions, and serious adverse events were captured from the time of administration until the final follow-up visit and followed up until resolution or stabilisation occurred.

Statistical analysis

The statistical analysis plan, including pre-planned interim analyses, was made available on the study registry before unblinding (appendix pp 17–42). Based on simulations performed in Fixed and Adaptive Clinical Trial Simulation version 6.2 (Berry Consultants), a maximum of 60 participants were required to detect the minimum effective dose between 0 ng/mL and 20 ng/mL to prevent streptococcal pharyngitis, with a power of more than 80%. Primary, secondary, and exploratory endpoints were represented by binary variables, with 0 indicating the endpoint was met (eg, diagnosis of clinical pharyngitis) and 1 indicating that it was not. Using

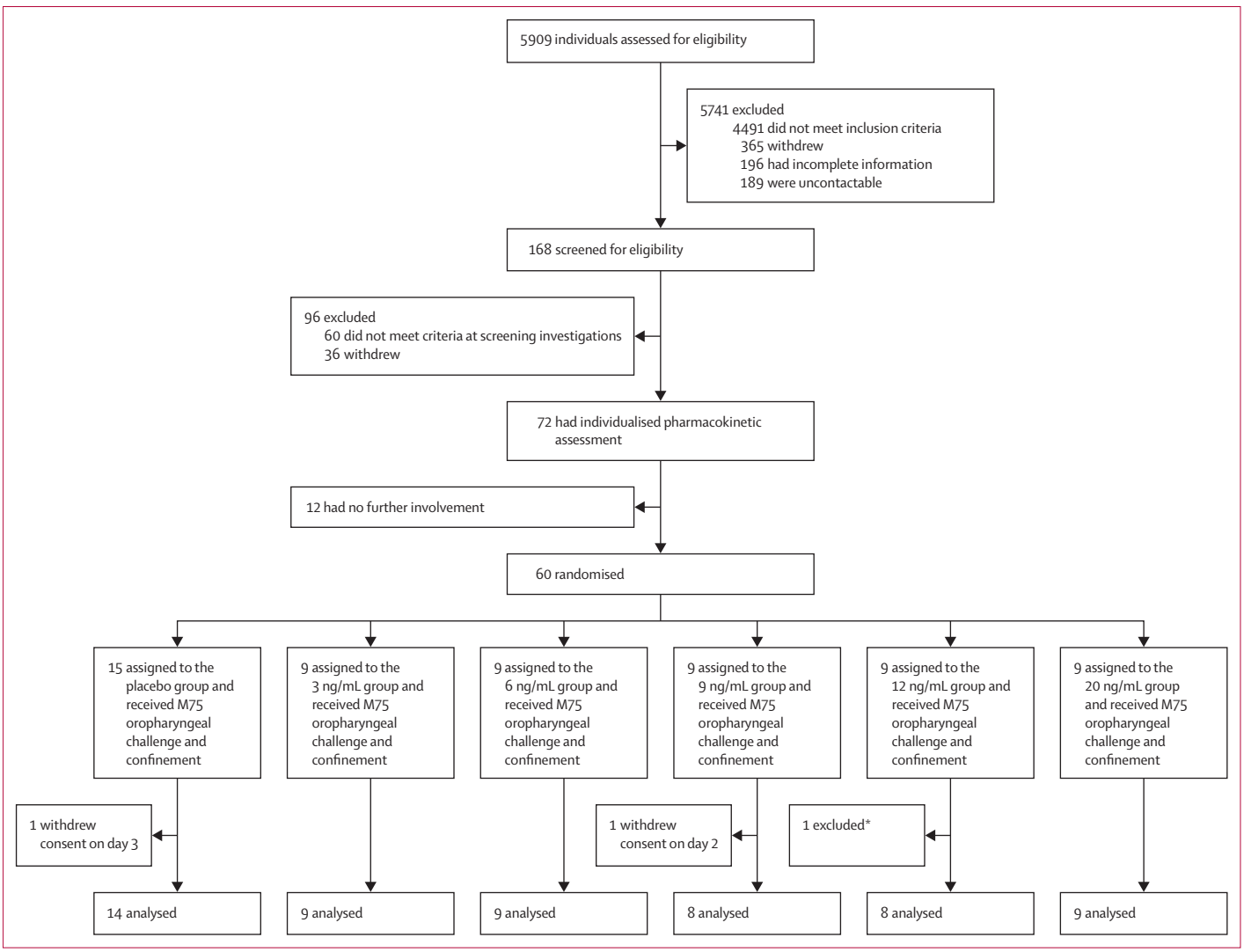

Figure 1: **Trial profile**
M75=*emm*75 strain of *Streptococcus pyogenes*. *Discharged on day 4 due to a tonsillolith erroneously interpreted as evidence of severe exudative pharyngitis in the absence of clinical symptoms.

Bayesian methods, a four-parameter binary E_{max} (dose–response effect) model (where E represents effect) was fitted to each endpoint (R package dreamer).[27] Priors were defined on the log scale for minimum response (E_0), maximum response (E_{max}), concentration that produces 50% of maximum effect (EC_{50}) and the steepness of the concentration–response curve (Hill parameter, N). However, the original priors appeared to be strongly informative and more weakly informative priors were defined post hoc for the concentration–response model for all endpoints as $E_0 \sim N(\text{logit}(0{\cdot}15),1^2)$, $E_{max} \sim N(\text{logit}(0{\cdot}99),0{\cdot}5^2)$, $EC_{50} \sim N(\log(9),1^2)$ and $N \sim N(1,5)$. For completeness, analyses are reported using the post-hoc priors and prespecified priors (appendix p 43). For each endpoint, the posterior mean concentration–response curve and 95% credible intervals (CrIs) were overlaid with response data for each allocated treatment group. A 95% CrI based on the posterior distribution was calculated for the minimum penicillin concentration required for a target clinical outcome of at least 90%.

Role of the funding source
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
Between Aug 23, 2022, and July 31, 2023, a total of 5909 adults were contacted and pre-screened for eligibility. Of these, 168 (2·8%) proceeded to screening, 72 (42·9%) of whom met eligibility criteria and had individualised penicillin pharmacokinetic dose-finding assessments (figure 1).

60 of these participants progressed and were randomly assigned and challenged with *emm*75 *S pyogenes* (table 1). Of these, 57 (95%) completed the inpatient challenge per

	Placebo (n=15)	3 ng/mL (n=9)	6 ng/mL (n=9)	9 ng/mL (n=9)	12 ng/mL (n=9)	20 ng/mL (n=9)
Included in analysis	14 (93%)	9 (100%)	9 (100%)	8 (89%)	8 (89%)	9 (100%)
Excluded from analysis	1 (7%)	0	0	1 (11%)	1 (11%)	0
Sex						
Female	9 (60%)	5 (56%)	4 (44%)	5 (56%)	6 (67%)	6 (67%)
Male	6 (40%)	4 (44%)	5 (56%)	4 (44%)	3 (33%)	3 (33%)
Age, years	25·0 (3·8; 18·9–31·5)	30·7 (5·3; 20·2–38·4)	26·1 (4·8; 20·9–34·5)	25·0 (5·2; 18·7–34·3)	25·6 (5·6; 18·2–36·1)	27·0 (4·5; 19·4–33·4)
BMI, kg/m^2	24·0 (3·6; 18·5–31·4)	23·4 (4·0; 18·4–28·5)	25·4 (2·9; 21·2–29·6)	25·2 (4·8; 19·5–31·9)	22·8 (3·8; 20·0–31·8)	25·7 (3·2; 21·2–31·2)

Data are n (%) or mean (SD; range).

Table 1: **Baseline characteristics**

protocol and were included in the analysis. Three (5%) participants were excluded from analysis; one (2%) was discharged early (day 4, assigned to the 12 ng/mL group) due to a tonsillolith interpreted as evidence of severe exudative pharyngitis in the absence of clinical symptoms (error in endpoint assessment determined before unblinding), and two (3%) participants withdrew consent (one in the 9 ng/mL group on day 2 and one in the placebo group on day 3), without meeting the primary endpoint. All 60 participants attended the 1-week and 1-month outpatient visits. Actual mean steady-state plasma penicillin concentrations aligned well with the allocated target levels (figure 2) and there was minimal within-person variability in steady-state concentrations during the confinement, as measured by acceptable coefficients of variation (table 2).

The primary clinical pharyngitis endpoint was met by 16 (27%) participants: eight (57%) of 14 in the placebo group, four (44%) of nine in the 3 ng/mL target steady-state penicillin plasma concentration group, four (44%) of nine in the 6 ng/mL group, zero of eight in the 9 ng/mL group, zero of eight in the 12 ng/mL group, and zero of nine in the 20 ng/mL group (table 2). The highest mean penicillin concentration where pharyngitis was observed was 6·6 ng/mL (figure 2). The minimum plasma penicillin concentration at which at least 90% of participants did not develop clinical pharyngitis, based on the Bayesian E_{max} model, was 8·1 ng/mL (95% CrI 6·1–10·9; figure 3).

S pyogenes pharyngeal colonisation was detected in 33 (55%) participants (table 2). The minimum plasma penicillin concentration required to avoid colonisation in at least 90% of participants was 13·0 ng/mL (95% CrI 10·1–16·0).

Peak C-reactive protein exceeded 20 mg/L in 17 (28%) participants. 22 (37%) participants met the primary endpoint or had C-reactive protein greater than 20 mg/L (table 2). Of the six (10%) participants assigned to penicillin 9 ng/mL or higher that met the secondary endpoint, none had C-reactive protein greater than 20 mg/L. The minimum plasma penicillin concentration required to avoid C-reactive protein inflammatory response in at least 90% of participants was 7·6 ng/mL (95% CrI 5·8–10·4).

Significant streptococcal antibody responses were most commonly measured against deoxyribonuclease B, followed by *S pyogenes* cell envelope proteinase and streptolysin O (table 2; figure 4).

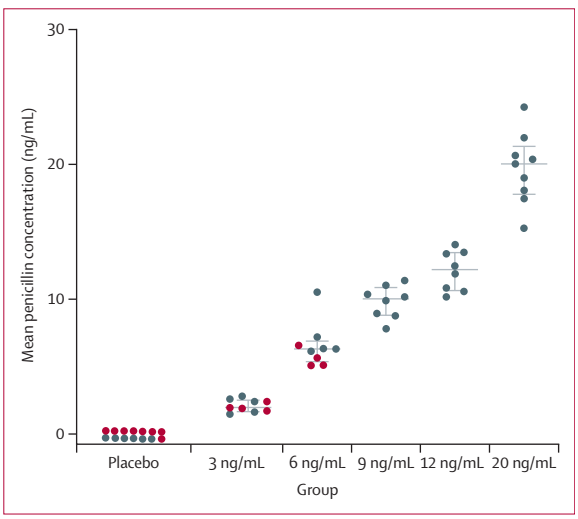

Figure 2: **Mean plasma penicillin concentration measured for individuals in each allocated group**

Steady-state concentrations were measured every 12 h from the commencement of the continuous infusion. Each dot reflects the average of these values for each individual. The grey horizontal lines and errors bars represent the median and IQR for each allocated group. Red dots represent the participants who met the primary endpoint of clinical pharyngitis.

There were 227 adverse events among 56 participants (appendix pp 2–7) on 210 occasions, including 131 (62%) that were possibly, probably, or definitely related to *S pyogenes* infection. Severity was graded as mild on 160 (76%) occasions and moderate on 50 (24%) occasions. There were no severe or serious adverse events related to *S pyogenes* infection or penicillin administration. The most common adverse events were sore throat (41 [18%] of 227 events), headache (31 [14%]), cannula site pain (19 [8%]), fever (nine [4%]), and myalgia (nine [4%]), all of which were more common in the placebo and lower penicillin dose groups, except for cannula site pain, which was reported evenly across groups. All adverse events related to participation had resolved by the 1-month outpatient visit.

The analyses using the priors specified in the statistical analysis plan produced similar results to the analyses using the less informative post-hoc priors (appendix p 30). Bayesian model diagnostics (ie, traceplots and Gelman's upper 95% confidence limit for the potential scale reduction factor and effective sample size) did not display any evidence

	Placebo (n=14)	3 ng/mL (n=9)	6 ng/mL (n=9)	9 ng/mL (n=8)	12 ng/mL (n=8)	20 ng/mL (n=9)
Treatment parameters						
Individual penicillin clearance, L/h	38 (12; 21–67)	34 (16; 22–71)	36 (9; 25–55)	39 (9; 26–52)	31 (4; 25–38)	35 (13; 21–60)
Individual mean steady-state penicillin concentration attained, ng/mL	0 (0; 0)	2·1 (0·6; 1·0–3·8)	6·8 (1·7; 3·6–11·8)	9·8 (1·5; 5·8–14·1)	12·1 (2·0; 8·0–16·5)	19·7 (3·1; 12·5–27·3)
Coefficient of variation for steady-state concentrations across individuals, (IQR)	0 (0–0)	17·4% (12·7–24·4)	11·4% (9·4–17·6)	9·5% (6·8–14·7)	12·2% (9·8–12·6)	9·5% (8·0–10·4)
Primary endpoint						
Pharyngitis diagnosed	8 (57%)	4 (44%)	4 (44%)	0	0	0
Secondary and exploratory endpoints						
Pharyngeal colonisation	14 (100%)	7 (78%)	6 (67%)	5 (63%)	0	1 (11%)
CRP >20 mg/L	8 (57%)	6 (67%)	3 (33%)	0	0	0
CRP >20 mg/L or primary endpoint met	11 (79%)	6 (67%)	5 (56%)	0	0	0
Serological responses (>0·2 $\log_{10}$ titre rise in antigen-specific IgG from baseline to 1-month outpatient visit)						
Deoxyribonuclease B	10 (71%)	6 (67%)	6 (67%)	2 (25%)	0	2 (22%)
Streptococcus pyogenes cell envelope proteinase	9 (64%)	6 (67%)	4 (44%)	2 (25%)	0	1 (11%)
Streptolysin O	8 (57%)	6 (67%)	2 (22%)	1 (13%)	1 (13%)	2 (22%)
Group A carbohydrate	4 (29%)	5 (56%)	4 (44%)	2 (25%)	2 (25%)	1 (11%)
Streptococcal C5a peptidase	3 (21%)	5 (56%)	2 (22%)	2 (25%)	0	0
S pyogenes adhesion and division protein	4 (29%)	5 (56%)	1 (11%)	1 (13%)	0	1 (11%)

Data are n (%) or mean (SD; range), unless otherwise specified. CRP=C-reactive protein.

Table 2: **Outcomes of study participants**

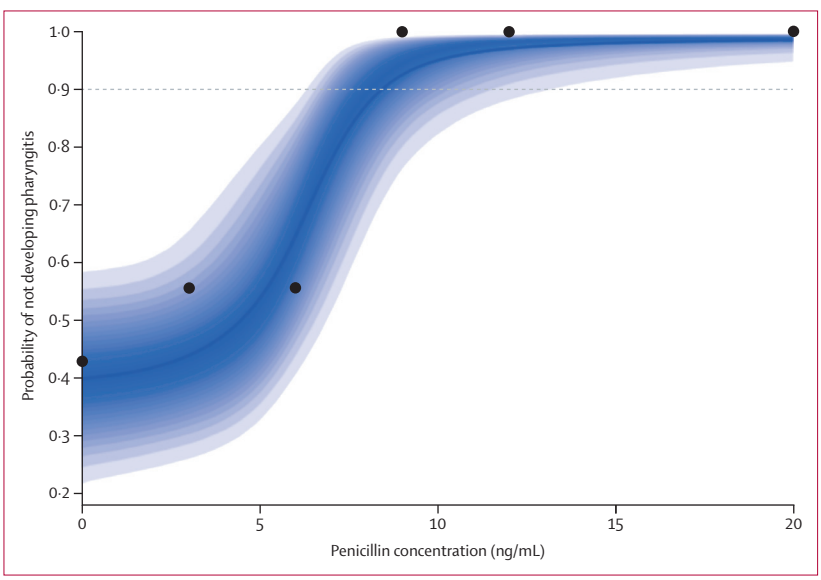

Figure 3: **Probability that clinical pharyngitis is avoided according to steady-state plasma concentration of penicillin following a human challenge with the *emm*75 strain of *Streptococcus pyogenes***
Observed proportions in allocated groups are shown (black dots). Bayesian concentration–response E_{max} curve is shown in blue: the solid line represents the posterior mean, and shaded areas represent symmetric credible intervals starting from 95% and decreasing by 5% increments. The grey dashed line indicates when the probability of avoiding clinical pharyngitis is 90%.

of lack of convergence to equilibrium for any posterior distributions.

Discussion

In this randomised, placebo controlled, *S pyogenes* challenge study, the minimum effective plasma penicillin concentration required to avoid pharyngitis in healthy adults was lower than the laboratory-derived MIC for the *emm*75 *S pyogenes* challenge strain. This finding helps to explain the effectiveness of regular benzathine benzylpenicillin injections in preventing recurrent rheumatic fever and progression of rheumatic heart disease, despite most patients not attaining traditionally accepted target concentrations. This finding will inform new secondary prophylaxis regimens with existing formulations and the target product profile for improved formulations of long-acting penicillin.

In the 70-year history of penicillin as the standard of care for secondary prophylaxis of rheumatic fever, our findings are the first randomised experimental human data supporting a target concentration to avoid streptococcal pharyngitis. No participants who attained a mean steady-state plasma concentration of 9 ng/mL or greater developed pharyngitis, suggesting a protective threshold lower than the historical 20 ng/mL target, and even lower than the in-vitro MIC for this strain (ie, 12 ng/mL). Observations that in-vitro subinhibitory levels of penicillin might alter *S pyogenes* infectivity through altered morphology, hydrophobicity, decreased M protein expression, and bacterial adhesion have been described previously, and might provide a mechanistic explanation of the in-vivo protection observed at subinhibitory levels in our study.[15,16]

Evidence of a protective threshold concentration lower than 20 ng/mL provides opportunities to improve the delivery and formulation of current long-acting penicillin preparations. Subcutaneous injections and infusions of benzathine benzylpenicillin, rather than traditional intramuscular administration, are absorbed more slowly to achieve lower peak concentrations over a longer period of time, with potential to increase the time between injections.[28] Emerging data suggest subcutaneous

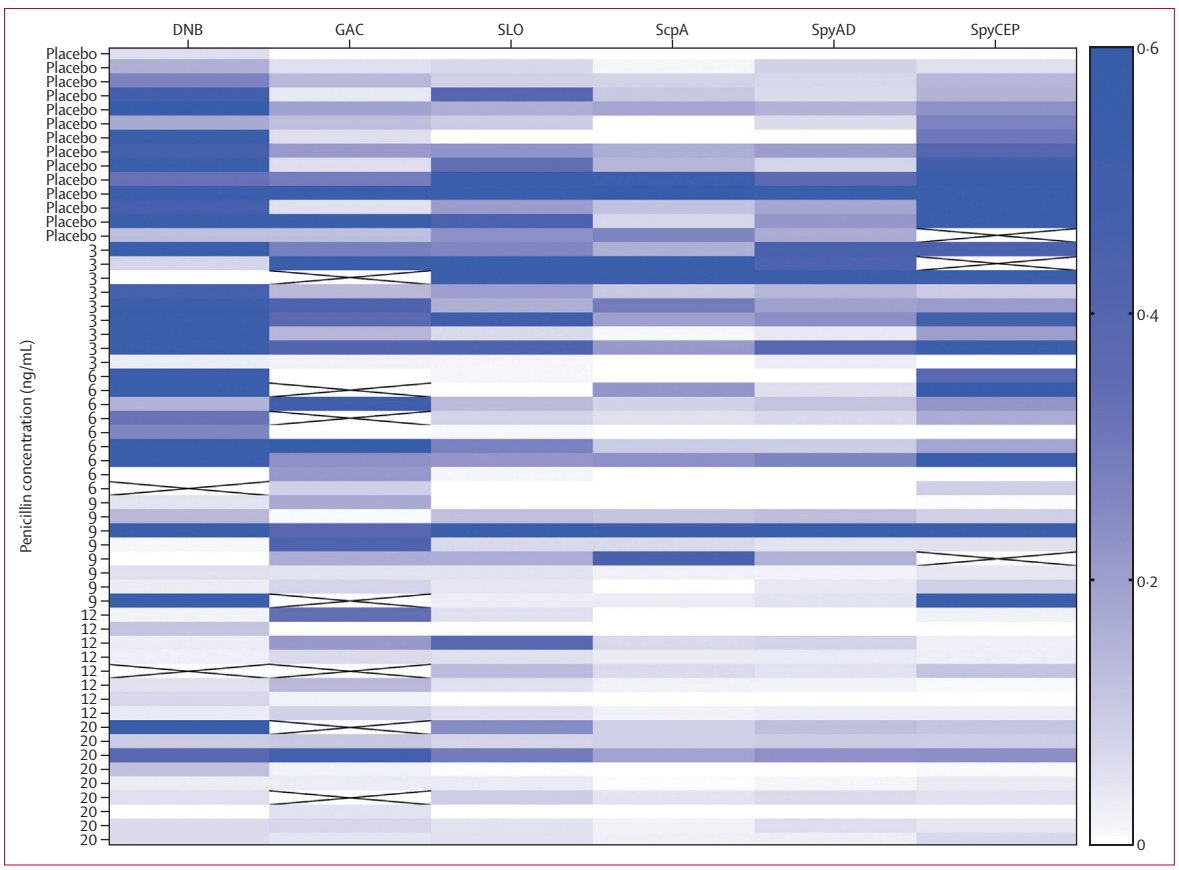

Figure 4: **Heatmap of participants' serological response to *Streptococcus pyogenes* antigens**
Multiplexed electrochemiluminescence assay, colour-coded according to magnitude of titre rise in >0·2 log_{10} (legend bar) between day –1 and day 28. Titre results with a high coefficient of variation are not mapped (cells marked with X). DNB=deoxyribonuclease B. GAC=group A carbohydrate. SLO=streptolysin O. ScpA=streptococcal C5a peptidase. SpyAD=*S pyogenes* adhesion and division protein. SpyCEP=*S pyogenes* cell envelope proteinase.

benzathine benzylpenicillin delivery is also less painful and more acceptable for people with rheumatic heart disease.[29–31]

A lower proportion of participants in the placebo group (57%) were diagnosed with pharyngitis compared with the initial study that established the human challenge model (85%),[20] which used a near-identical protocol (ie, participant screening, challenge inoculum, procedures, and case definition). Even with assessor training and the incorporation of a rapid molecular test, pharyngitis remains a clinical diagnosis primarily, and inter-assessor and intra-assessor variation in applying the standardised clinical case definition for primary endpoint assessment could not be wholly accounted for and might feasibly explain the lower attack rate. Among participants who did not meet the primary endpoint, there were an additional five participants (three of seven in the placebo group and two of five in the 6 ng/mL group), with a peak C-reactive protein of greater than 20 mg/L, which is evidence of a systemic inflammatory response suggestive of pharyngitis. Crucially, these clues to potential underdiagnosis were not found among participants in the 9 ng/mL, 12 ng/mL, and 20 ng/mL groups, supporting the overall finding of this trial. There could be opportunities to refine the case definition for use in future *S pyogenes* human challenge studies, incorporating an additional objective element such as a point-of-care C-reactive protein assay.

A key strength of this study was the accuracy and consistency of target attainment across the allocated target plasma penicillin concentrations of penicillin, enabled by individual pharmacokinetic assessments and analytical methods validated to extremely low concentrations. All study visits and assessments were completed by 57 (95%) of the 60 participants, with only two participants withdrawing during the inpatient phase, suggesting that participation was acceptable to most.

A limitation of this study was the modest sample size of 60 participants, which was adequate to detect the anticipated effect but not for finetuning the dose–response modelling. Almost 200 participants have participated in four previous *S pyogenes* human challenge studies since 1973, and only one study had more than 60 participants.[20,32–34]

Another limitation was the experimental infection of healthy adults with the goal of improving secondary prophylaxis, which is delivered predominantly to children and young adults with previous rheumatic fever or rheumatic heart disease, who might have different immunity to

S pyogenes and different susceptibility to pharyngitis and colonisation. The rationale for selection of the *emm*75 *S pyogenes* strain has previously been described in detail.[22] Although there is ongoing interest in rare *S pyogenes* isolates with relatively reduced susceptibility to some penicillins, *S pyogenes* remains universally susceptible to penicillin and the threshold for protection in this trial is likely generalisable to most other strains.[35,36]

Finally, variation in penicillin concentrations in tonsillar tissue or saliva could also account for variation in clinical response, but considering the invasiveness of sampling, technical challenges in obtaining standardised samples, and development of an assay with a different tissue matrix, plasma penicillin concentrations remain a practical, easily collectable correlate of protection that can be consistently and reproducibly measured.

Using a human challenge model of *emm*75 *S pyogenes* pharyngitis, we determined that the minimum steady-state plasma benzylpenicillin concentration required to reduce the risk of pharyngitis to less than 10% has a mean of no more than 9 ng/mL (95% CrI 6·1–10·9), much lower than the conventional target of 20 ng/mL, and lower than the in-vitro MIC for the strain. This finding supports observational evidence suggesting lower concentrations might be sufficient and boosts efforts to develop improved longer-acting penicillin formulations for secondary prevention of rheumatic heart disease.

Contributors

TKH, JO, JC, JK, JAM, JSM, ACS, RKB, TLS, and LM conceived and designed the study. SS, MP-S, OY, and KTB conducted pharmaceutical and pharmacokinetic evaluations. JAM and TLS completed the statistical analysis. TKH, SLE, and LH were responsible for the clinical conduct of the trial. KA, MM, and AF completed quantitative PCR and serological testing. TKH and LM wrote the first draft of the manuscript, with input from JO, JC, ACS, and JSM. All authors had full access to the data in the study and had final responsibility for the decision to submit for publication. TKH and JAM accessed and verified the underlying data reported in the manuscript.

Declaration of interests

LM is currently supported by Medical Research Future Fund Emerging Leadership Fellowship 2 (APP 1197177). JC is currently supported by a National Health and Medical Research Council Investigator Grant (1173874). All other authors declare no competing interests.

Data sharing

Outcome data with accompanying pharmacokinetic data will be made available on reasonable request made to the corresponding author. The protocol is available on the Australian New Zealand Clinical Trials Registry (registration number ACTRN12621000751875). Full details on statistical details are provided in the accompanying statistical analysis plan (appendix pp 17–42).

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