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Genomic investigation and clinical correlates of the *in vitro* β-lactam: $NaHCO_3$ responsiveness phenotype among methicillin-resistant *Staphylococcus aureus* isolates from a randomized clinical trial

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ABSTRACT $NaHCO_3$ responsiveness is a novel phenotype where some methicillin-resistant *Staphylococcus aureus* (MRSA) isolates exhibit significantly lower minimal inhibitory concentrations (MIC) to oxacillin and/or cefazolin in the presence of $NaHCO_3$. $NaHCO_3$ responsiveness correlated with treatment response to β-lactams in an endocarditis animal model. We investigated whether treatment of $NaHCO_3$-responsive strains with β-lactams was associated with faster clearance of bacteremia. The CAMERA2 trial (Combination Antibiotics for Methicillin-Resistant *Staphylococcus aureus*) randomly assigned participants with MRSA bloodstream infections to standard therapy, or to standard therapy plus an anti-staphylococcal β-lactam (combination therapy). For 117 CAMERA2 MRSA isolates, we determined by broth microdilution the MIC of cefazolin and oxacillin, with and without 44 mM of $NaHCO_3$. Isolates exhibiting ≥4-fold decrease in the MIC to cefazolin or oxacillin in the presence of $NaHCO_3$ were considered "$NaHCO_3$-responsive" to that agent. We compared the rate of persistent bacteremia among participants who had infections caused by $NaHCO_3$-responsive and non-responsive strains, and that were assigned to combination treatment with a β-lactam. Thirty-one percent (36/117) and 25% (21/85) of MRSA isolates were $NaHCO_3$-responsive to cefazolin and oxacillin, respectively. The $NaHCO_3$-responsive phenotype was significantly associated with sequence type 93, SCC*mec* type IVa, and *mecA* alleles with substitutions in positions −7 and −38 in the regulatory region. Among participants treated with a β-lactam, there was no association between the $NaHCO_3$-responsive phenotype and persistent bacteremia (cefazolin, P = 0.82; oxacillin, P = 0.81). In patients from a randomized clinical trial with MRSA bloodstream infection, isolates with an *in vitro* β-lactam-$NaHCO_3$-responsive phenotype were associated with distinctive genetic signatures, but not with a shorter duration of bacteremia among those treated with a β-lactam.

KEYWORDS methicillin-resistant *Staphylococcus aureus*, MRSA, bloodstream-infections, sodium bicarbonate ($NaHCO_3$), β-lactams, bacterial genomics

Staphylococcus aureus bloodstream infections (BSI) are common and result in high morbidity and mortality (1). Whereas the treatment of choice for methicillin-susceptible *S. aureus* (MSSA) infections are β-lactams (2), treatment options for methicillin-resistant *S. aureus* (MRSA) BSIs are limited by their inherent resistance *in vitro* to most β-lactams.

The current first-line therapy for MRSA BSI is vancomycin or daptomycin (3). β-lactams are considered superior to vancomycin for the treatment of MSSA BSI (2) and have a

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Arnold S. Bayer passed away during the preparation of this manuscript.

The authors declare no conflict of interest.

See the funding table on p. 14.

This work is dedicated to the memory of Professor Arnold S. Bayer, who has pioneered much of the work to date on $NaHCO_3$ responsiveness in MRSA. More importantly, Professor Bayer has acted as a kind, generous, and highly respected mentor and colleague to many in the staphylococcal research and clinical community.

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good safety profile. β-lactams are generally considered ineffective against MRSA, which harbors a modified penicillin-binding protein 2a that has low affinity to most β-lactams and leads to high minimum inhibitory concentrations (MIC) to this class of antibiotics. However, β-lactams may have sub-MIC effects that result in faster clearance of bacteremia by enhancing activity of cationic peptides of the innate immune system (4). They may also act synergistically with vancomycin and daptomycin *in vitro* against selected MRSA strains (5). In some cases, synergy had been attributed to the "see saw effect" (6, 7) where increased resistance to vancomycin or daptomycin is associated with lower MICs to some β-lactams, but synergy has also been reported even in the absence of the "see saw effect" (8).

Recently, a $NaHCO_3$-β-lactam-responsive phenotype has been described, where many clinical MRSA isolates exhibit substantially lower MICs to the anti-staphylococcal β-lactams, cefazolin (CFZ) and oxacillin (OX), when grown in Mueller Hinton broth (MHB) supplemented by 44 mM $NaHCO_3$ (9, 10). This $NaHCO_3$-containing media is thought to better represent the host environment during actual infection than standard MHB (10). These lower MICs to β-lactams in the presence of $NaHCO_3$ supplementation were sometimes below the drug's clinical breakpoints and correlated with increased killing *in vitro*, as well as salutary treatment responses to such β-lactams *ex vivo* and *in vivo* infective endocarditis models (9, 11).

These $NaHCO_3$-responsive strains were shown to have reduced *mecA* expression, PBP2a production (9), and lower expression of other genes involved in cell wall biosynthesis (12) when grown in the presence of $NaHCO_3$. In media containing $NaHCO_3$, oxacillin and cefazolin also synergized with the host immune peptide LL-37, which led to reduced survival of the responsive strains compared to non-responsive strains (9). Ersoy et al. (13) found that the $NaHCO_3$-responsive phenotype can be predicted by combining a simple phenotypic test, the amoxicillin-clavulanate disc diffusion assay, together with two genotypic markers, the *spa* type, and an allele of the *mecA* gene containing a substitution in the ribosome binding site (RBS; position −7 G to T) and an amino acid substitution at position 246 (E246G) [i.e., the "*mecA*-susceptible" genotype as defined by Harrison et al. (14)]. However, they tested only 60 isolates belonging to three prevalent genetic backgrounds circulating in the USA (clonal complex 1, 5, 8), and the clinical implications of this phenotype were not assessed.

There is little clinical evidence to guide which patients, if any, should receive β-lactams as part of their treatment for MRSA infections. The most recent prospective data come from the CAMERA2 trial (15) (Combination Antibiotics for Methicillin-Resistant *Staphylococcus aureus*) which was an open-label, randomized trial that assessed the addition of β-lactam (usually flucloxacillin, cloxacillin, or cefazolin) to vancomycin compared to standard monotherapy (usually vancomycin) in patients with MRSA bacteremia. While the study was stopped prematurely due to higher rates of acute kidney injury in the vancomycin plus β-lactam group, rates of persistent bacteremia were reduced in the combination group.

We hypothesized that $NaHCO_3$ responsiveness might contribute to the above salutary β-lactam response in CAMERA2. Our aim in the current study was to characterize the $NaHCO_3$-responsive phenotype *in vitro* among a large collection of MRSA BSI isolates from the CAMERA2 trial that represent a diverse genetic and geographic background, as well as to look for a genetic signature of such strains. We investigated for an association between the $NaHCO_3$-responsive vs $NaHCO_3$-non-responsive phenotypes and persistent bacteremia among CAMERA2 trial participants who were treated with a β-lactam.

MATERIALS AND METHODS

Study design, participants, and MRSA BSI isolates

The CAMERA2 trial has been previously published (15). Briefly, this was an open-label, randomized trial, comparing the treatment of MRSA BSI with standard treatment alone (vancomycin or daptomycin) to a combination of standard treatment with an

anti-staphylococcal β-lactam (either flucloxacillin or CFZ). The study enrolled 352 adult participants (>18) with MRSA BSI in Australia, New Zealand, Singapore, and Israel between 2015 and 2018.

MRSA isolates

Three hundred thirty MRSA index blood culture isolates were collected as part of the CAMERA2 trial. We selected 117 isolates for the $NaHCO_3$-responsiveness studies, restricting our selection to community-onset infections, and those from the most prevalent multilocus sequence types (MLSTs), namely ST 5, 22, 45, and 93, to facilitate comparisons between isolates of different genetic backgrounds (Fig. 1). There were 70 isolates from participants randomly allocated to combination therapy of vancomycin (n = 68) or daptomycin (n = 2) with β-lactams, including seven isolates from participants with persistent bacteremia, defined as ongoing bacteremia at study day 5. There were 68 isolates from participants receiving monotherapy with vancomycin or daptomycin, and 47 were chosen as a control population (all were treated with vancomycin). These included 17 isolates collected from participants that had persistent bacteremia, which were then matched to isolates from the same STs and were collected from participants without persistent bacteremia. These 47 isolates included 15 isolates which were sent to the Lundquist Institute, Torrance, California, USA, where they were tested for OX MICs and OX-$NaHCO_3$ responsiveness.

In vitro $NaHCO_3$ responsiveness

CFZ-$NaHCO_3$ responsiveness was tested for at the Doherty Institute, Melbourne, Victoria, Australia, while OX-$NaHCO_3$ responsiveness was assessed at the Lundquist Institute, Torrance, California, USA. CFZ-$NaHCO_3$ responsiveness was determined for all 117 isolates, and OX-$NaHCO_3$ responsiveness for 85 isolates (70 given combination therapy, 15 given monotherapy). CFZ (Alphapharm, Australia) and OX (Sigma-Aldrich, USA) MICs were determined by broth microdilution (BMD) according to Clinical and Laboratory Standards Institute standards (16). The isolates were grown on horse blood agar for 18–20 h and diluted to 1×10^8 CFU/mL into cation-adjusted Mueller Hinton broth (CA-MHB), then further diluted 1 in 100 into CA-MHB with or without $NaHCO_3$ (44 mM) to a concentration of 1×10^6 CFU/mL. BMD was performed in a 96-well round bottom plate (Costar). A volume of 50 µL CA-MHB with or without $NaHCO_3$ was transferred to all wells of columns 2 to 12. CA-MHB supplemented with antibiotic was added at a volume of 100 µL to column 1 and 50 µL was serially diluted twofold across the plate to column 11. The bacterial cell suspension was added to the plate at a volume of 50 µL to a final concentration of 5×10^5 CFU/mL. The plates were incubated at 35°C ± 2°C for 16–20 h in an ambient air incubator. All media used were supplemented with 100 mM Tris maintained at pH 7.2 (7.2–7.5). When performing OX MICs, media was also supplemented with 2% NaCl, and incubation time was extended to 24 h. The MIC was recorded as the antibiotic concentration of the well displaying no visible growth. Each isolate was tested in duplicate; a third replicate was performed if there was discordance between the first two BMD results. "$NaHCO_3$ responsiveness" was defined as ≥4-fold decrease in the MIC measured by BMD when grown in MHB media supplemented by 44 mM $NaHCO_3$ vs standard MHB (9). This latter $NaHCO_3$ concentration reflects deep tissue levels and has been used in all our prior studies of this phenotype (9–13, 17–21). All MIC testing was performed "blinded" as to the clinical treatment groups and ultimate outcomes of patients.

In vitro susceptibility to vancomycin and daptomycin

Vancomycin and daptomycin MICs for each bacterial isolate were determined by Sensititre broth microdilution (Thermo Fisher Scientific).

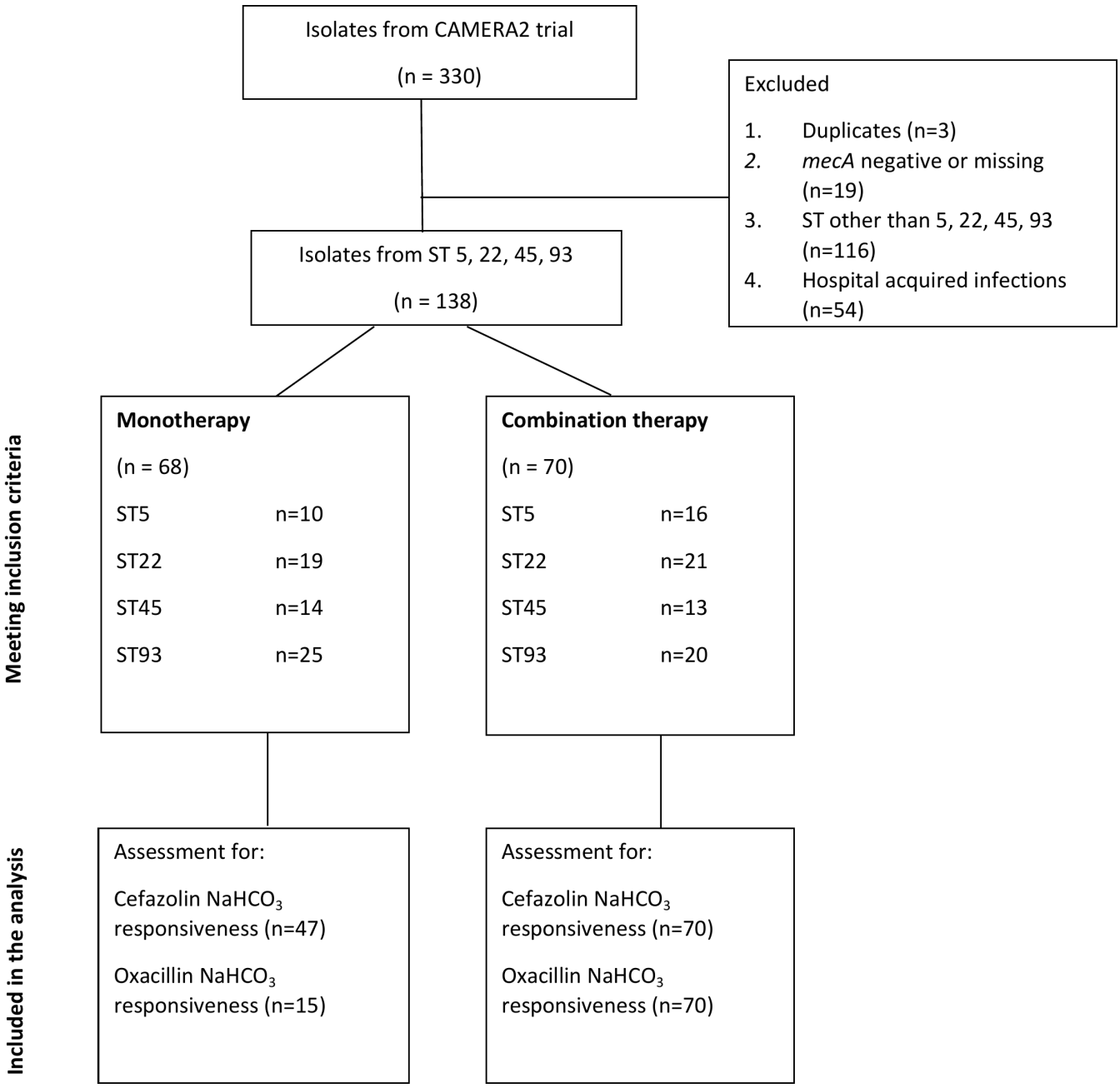

FIG 1 Isolate selection process. CAMERA2, Combination Antibiotics for Methicillin Resistant *Staphylococcus aureus*; ST, sequence type.

Genotypic markers of the $NaHCO_3$-responsive phenotype

All isolates have been previously whole genome sequenced using the Illumina Next Seq platform (15) and are publicly available under BioProjects PRJEB50796 and PRJEB63381. Quality control, assembly of short reads, and assembly annotation were performed as described elsewhere (22). MLSTs were determined *in silico* using MLST version 2.16.4 (https://github.com/tseemann/mlst). We determined the *SCCmec* type using Staphopia SCCmec (23). Resistance genes were detected using abriTAMR (24).

To identify mutations in the *mecA* locus (gene and regulatory region), reads were mapped to reference genome Sa FPR3757 and variants were determined with Snippy, version 4.6 (https://github.com/tseemann/snippy). In addition, *mecA* loci were extracted from the assemblies using BLAST and aligned using MUSCLE (25). We modified a recently

published genotypic scheme by Harrison et al. (14) to describe *mecA* haplotypes sharing a combination of single nucleotide polymorphisms (SNPs) (Fig. 2 and Table S4). In the original report by Harrison et al., these haplotypes (resistant 1 and 2 and susceptible 1–4) were correlated with susceptibility to the combination of penicillin and clavulanic acid, and with *mecA* expression levels as measured by reverse transcription-quantitative PCR (qRT-PCR).

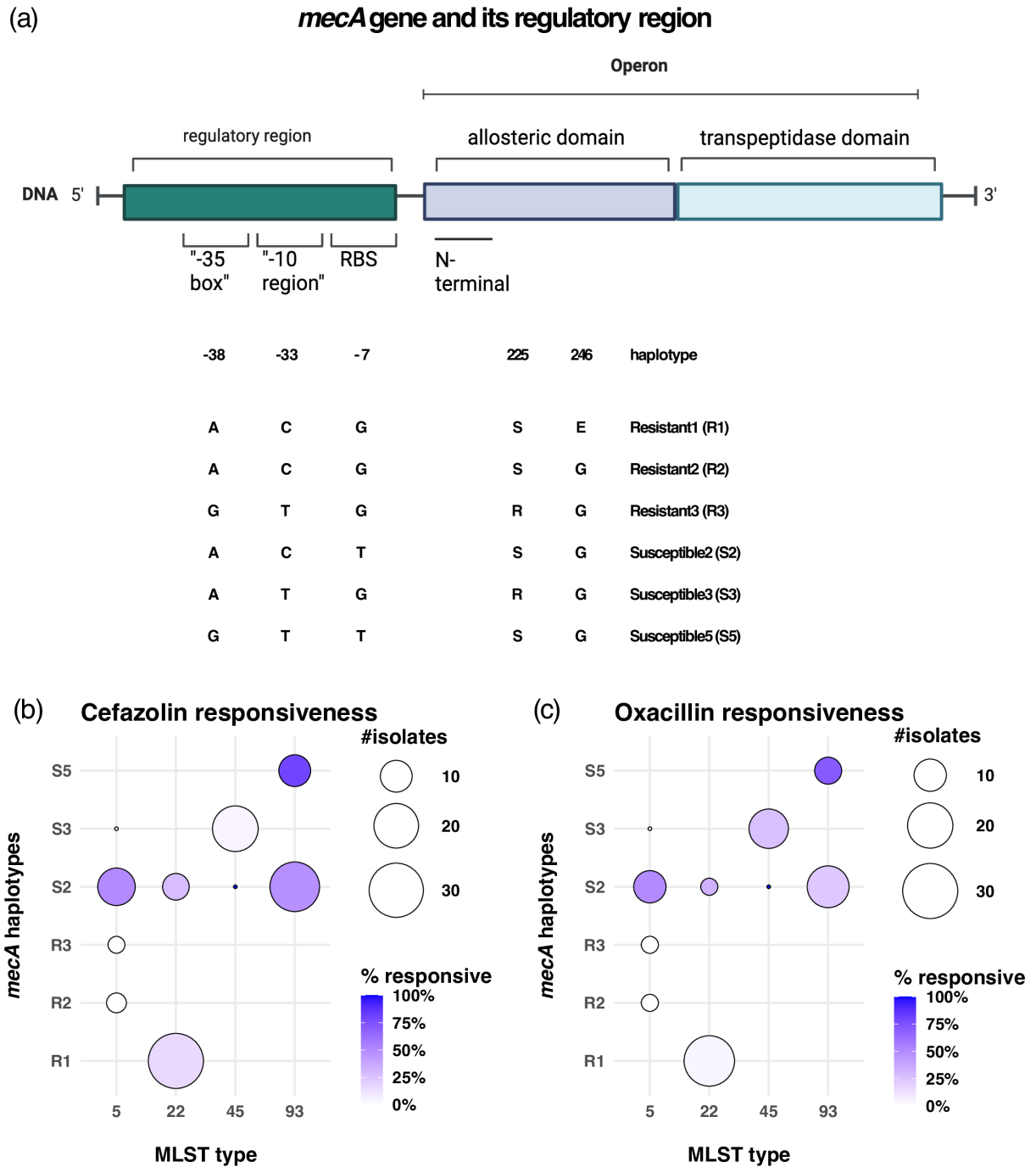

FIG 2 (a) *mecA* haplotypes. Scheme of the *mecA* gene and its regulatory region. SNPs in the regulatory region and amino acid substitutions in the PBP2a protein were used to annotate *mecA* haplotypes. (b and c) Balloon plot of the intersection between ST, *mecA* haplotype, and (b) cefazolin-$NaHCO_3$ responsiveness and (c) oxacillin-$NaHCO_3$ responsiveness. Cefazolin- and oxacillin-$NaHCO_3$ responsiveness are defined as a fourfold or greater decrease in the MIC to that agent in the presence of 44 mM of $NaHCO_3$. RBS, ribosome binding site; MLST, multilocus sequence type; MIC, minimal inhibitory concentration. Created with BioRender.com.

Using Snippy, we generated a core genome alignment including all sites with ≥90% coverage across the 117 isolates. We used IQ-tree, version 2.1.2, to infer a maximum likelihood phylogeny that was visualized using ggtree version 3.10.0 (26).

Clinical outcomes

We examined for associations between $NaHCO_3$ responsiveness to either CFZ and/or OX *in vitro* with the following clinical trial outcomes: persistent bacteremia at trial day 5 (primary outcome); 30- and 90-day all-cause mortality (secondary outcomes).

Statistical analysis

Means are presented with standard deviations and medians with interquartile (25%–75%) range (IQR). Categorical variables were compared using χ^2 test and calculating the odds ratios (OR) with 95% confidence intervals (95% CI). Continuous variables with a normal distribution were compared using a Student's *t*-test, and variables with a skewed distribution were compared using a Mann-Whitney U-test.

For participants that were allocated to β-lactam treatments, we assessed the association between the outcome, persistent bacteremia, and its predictors (including the $NaHCO_3$-responsive phenotype) and confounders using simple logistic regression. Variables that were associated with persistent bacteremia (*P*-value <0.2) were planned to be included in a multiple logistic regression model. Potential confounders and other risk factors for persistent bacteremia included age, comorbidities (Charlson Comorbidity Index), and the final diagnosis of source of infection [grouped into three risk groups (27): high risk—endovascular, pneumonia, abdominal, central nervous system; and non-high risk—grouping together either intermediate risk—osteoarticular, soft tissue, primary BSI; and low risk—intravenous catheters, urinary tract infections].

Statistical analysis was done in Stata version 17. Figures were generated in Stata version 17 and R version 2023.03.0+386 using the package ggplot (version 3.4.4) and ggtree (version 3.10.0).

RESULTS

Of the originally collected 330 isolates from the CAMERA2 trial, 117 isolates met the eligibility criteria for this study (Fig. 1). These 117 isolates were collected from participants in Australia/New Zealand (68%), Singapore (22%), and Israel (9%), and belonged to ST 5 (19%), 22 (32%), 45 (19%), and 93 (30%). The phylogenetic tree along with associated metadata (Fig. 3) shows the population structure of the data set, confirming the four clonal lineages with a further splitting of ST 45 into two distinct sub-clades as previously observed (28).

$NaHCO_3$-responsive phenotype vs genetic background

The MICs of CFZ and OX were determined in the absence vs presence of 44 mM of $NaHCO_3$ for 117 and 85 MRSA isolates, respectively (Fig. 1). CFZ and OX MICs ranged from 2 to 256 mg/L (median 64 mg/L, IQR 32–256) and 0.25 to 256 mg/L (median 32 mg/L, IQR 16–64), respectively (Fig. 4). Of the strains tested, 31% (36/117) and 25% (21/85) were CFZ-$NaHCO_3$- and OX-$NaHCO_3$-responsive, respectively. Of note, these proportions for $NaHCO_3$ responsiveness for CFZ are substantially lower than that observed in US BSI isolates (21). Of the 85 isolates that were tested for both CFZ-$NaHCO_3$ and OX-$NaHCO_3$ co-responsiveness, both tests were in agreement in 76% of isolates; 13 isolates (15%) were responsive to both antibiotics, 58 (61%) were non-responsive to both agents, and 20 (24%) isolates had discordant results.

We assessed whether the $NaHCO_3$-responsive phenotype was a manifestation of the "see-saw effect" where responsive strains are more peptide or daptomycin resistant (7). The daptomycin MIC distribution for the 117 isolates was 0.25 (n = 84), 0.5 (n = 29), and 1 (n = 4)(Table S1). Unexpectedly, we found that the daptomycin MIC was significantly lower for CFZ-$NaHCO_3$-responsive (median MIC 0.25 mg/L, IQR 0.25–

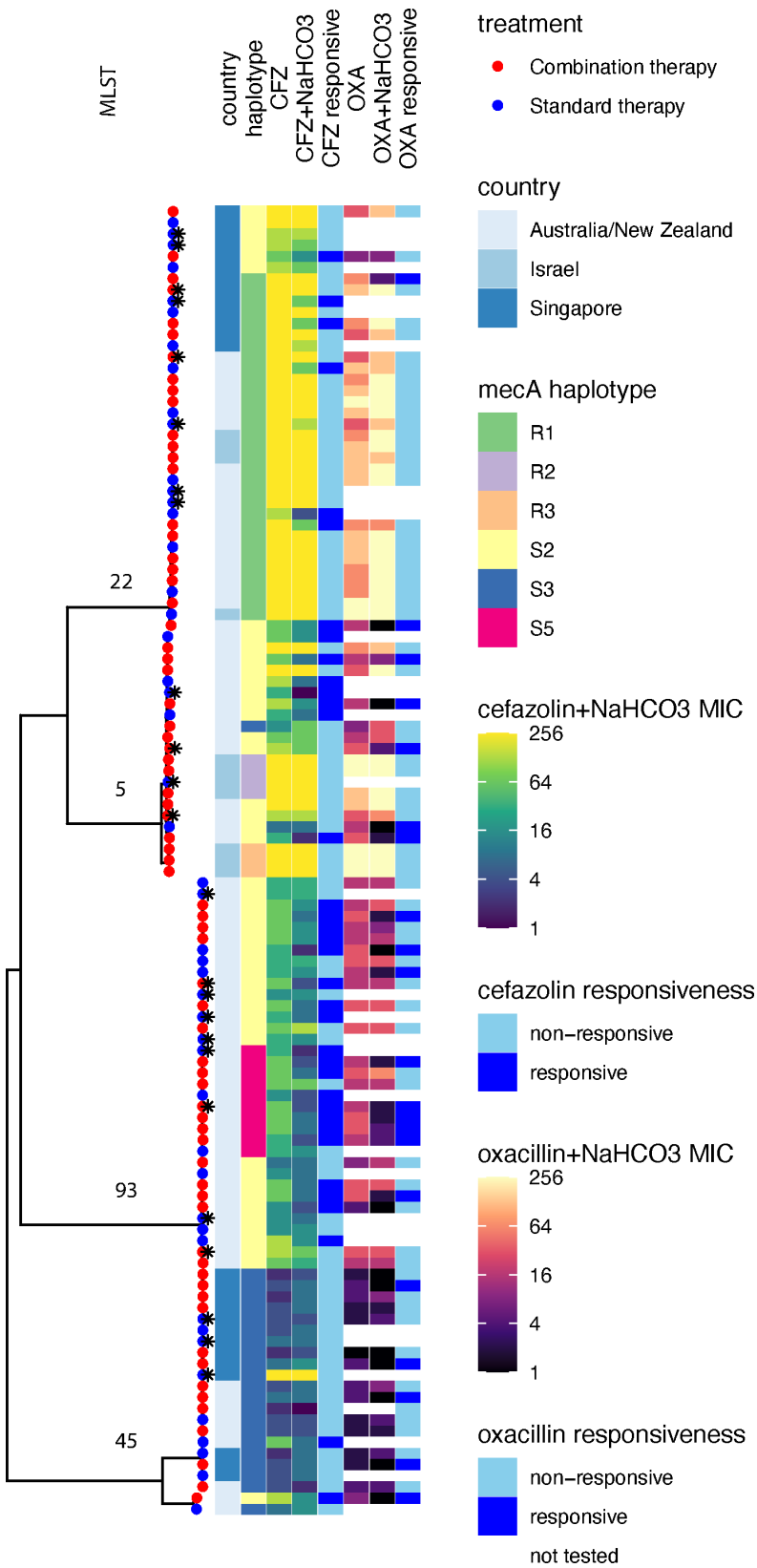

FIG 3 Phylogenetic tree. Maximum likelihood phylogenetic tree of 117 MRSA isolates built from a core genome SNP alignment. STs are shown on the branches. *mecA* haplotypes (R1, R2, R3, S2, S3, S5) were annotated based on combinations of SNPs in the *mecA* regulatory region and SNPs in the coding region

(Continued on next page)

FIG 3 (Continued)

resulting in amino acid substitution. The MICs of cefazolin and oxacillin alone, and in the presence of 44 mM of $NaHCO_3$, are presented along with the cefazolin-$NaHCO_3$- and oxacillin-$NaHCO_3$-responsive phenotype, which is defined as a greater than fourfold decrease in the MIC measured in the presence compared to the absence of $NaHCO_3$. Isolates collected from participants with persistent bacteremia are marked with an asterisk (*). MRSA, methicillin-resistant *Staphylococcus aureus*; SNP, single nucleotide polymorphism; ST, sequence type; MIC, minimal inhibitory concentration; CFZ, cefazolin; OXA, oxacillin.

0.25) than non-responsive strains (median MIC 0.25 mg/L, IQR 0.25–0.5) (P = 0.001) and similar for OX-$NaHCO_3$-responsive (median MIC 0.25 mg/L, IQR 0.25–0.25) and non-responsive strains (median MIC 0.25 mg/L, IQR 0.25–0.25) (P = 0.37). The vancomycin MIC was <2 mg/L in 113/117 isolates and the MIC = 2 mg/L in 4/117 isolates; 0/4 and 1/2 were CFZ-$NaHCO_3$- and OX- $NaHCO_3$-responsive, respectively.

The $NaHCO_3$-responsive phenotype was associated with several specific genotypic markers (Fig. 4). Isolates belonging to ST 93 had higher rates of $NaHCO_3$ responsiveness [57% (20/35) to CFZ and 38% (9/24) to OX] vs the other three STs in our collection (ST 5, 22 and 45) (P < 0.001 for CFZ-$NaHCO_3$ and P = 0.02 for OX-$NaHCO_3$). Similarly, SCC*mecIVa* was associated with a β-lactam-responsive phenotype vs other cassette types (Table S1), but this was highly correlated with the ST (34/38 of isolates harboring the SCC*mecIVa* cassette type belonged to ST93).

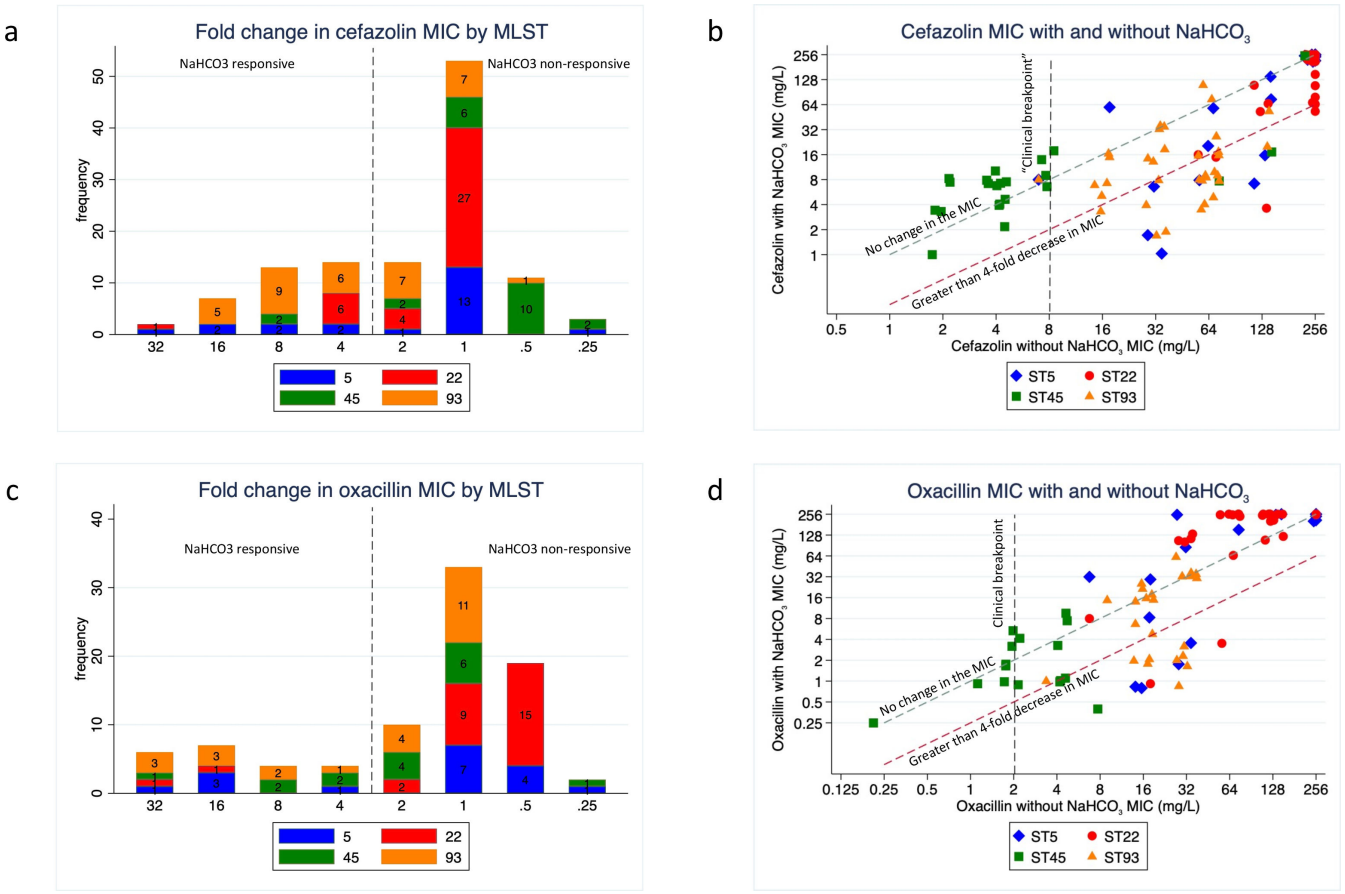

FIG 4 Cefazolin-$NaHCO_3$ and oxacillin-$NaHCO_3$ responsiveness by MLST. (a and c) fold-change in the MIC of cefazolin (a) and oxacillin (c) in the presence of $NaHCO_3$ compared to MHB alone colored by MLST. The $NaHCO_3$-responsive phenotype is defined by a greater than fourfold reduction in the MIC in the presence of $NaHCO_3$ (e.g., from 16 mg/L on MHB to 4 mg/L on MHB with the addition of 44 mM $NaHCO_3$). (b and d) scatter plot of MIC (mg/L) values of (b) cefazolin and (d) oxacillin with and without $NaHCO_3$ by BMD. Panels b and d illustrate the relationship between MIC with and MIC without $NaHCO_3$, with isolates in the lower right corner (below the second dashed line) representing $NaHCO_3$-responsive groups. MIC, minimal inhibitory concentration; MLST, multilocus sequence type; ST, sequence type; MHB, Mueller Hinton Broth; BMD, broth microdilution.

The *mecA* gene and its regulatory areas from the reference MRSA strain BPH2900 (ST 22) were examined against our MRSA isolates for SNPs. The regulatory area of the *mecA* gene had three previously described SNPs at positions −38 (A→G), −33 (C→T) in the "−10 box," and −7 (G→T) in the RBS that were present in 11%, 30%, and 49% of isolates, respectively (Table S1). The coding region of the *mecA* gene had three SNPs that led to amino acid substitutions at positions N146K, S225R, and E246G in the PBP2a protein, and these were present in 3%, 21%, and 73% of isolates, respectively. MRSA strains harboring a *mecA* allele with a G-to-T substitution at position −7 in the RBS or an A-to-G substitution in position −38 in the promoter had higher rates of the "responsive" phenotype (Table S1). Combinations of these SNPs were used to create a genotypic schema of "resistant" and "susceptible" haplotypes based on prior work done by Harrison et al. (14) with an addition of a new "susceptible" haplotype, S5 (Fig. 2). Isolates with a *mecA* "susceptible" haplotype had an odds ratio of 4.3 (95% CI 1.5–12.1) and 17.6 (95% CI 2.2–139.5) to have a $NaHCO_3$-responsive phenotype to CFZ and OX, respectively, vs isolates with a *mecA*-"resistant" haplotype (Table S1). Figure 2 shows the correlation between the ST, the *mecA* haplotype, and the CFZ-$NaHCO_3$- and OX-$NaHCO_3$-responsive phenotypes. Isolates harboring the S5 haplotype of the *mecA* gene were more likely to have a CFZ- (unadjusted odds ratio (uOR) 20.8, 95% CI 3.4–128.5) and OX- (uOR 60, 95% CI 4.5–797) $NaHCO_3$-responsive phenotype vs the *mecA* R1 haplotype.

Association between the $NaHCO_3$ phenotype and clinical outcomes of participants treated with β-lactam combination regimens

To determine if "$NaHCO_3$ non-responsiveness" was associated with persistent bacteremia among participants allocated to combination therapy with a β-lactam, we compared the rates of persistent bacteremia between such participants who had infections caused by $NaHCO_3$-responsive vs non-responsive strains. Blood cultures were persistently positive at trial day 5 in 10% (7/68) of participants who received a β-lactam (data were missing for two participants), and this rate was similar for participants who had CFZ-$NaHCO_3$-responsive and -non-responsive strains (9% and 11%, respectively; $P = 0.82$), or OX-responsive and -non-responsive strains (12% and 10%, respectively; $P = 0.81$) (Fig. 5; Table S2).

When comparing participants with high-risk vs non-high-risk (intermediate and low) infections, $NaHCO_3$-responsive isolates were overrepresented among high-risk infections [CFZ-$NaHCO_3$-responsive in 41% of high risk vs 27% non-high risk ($P = 0.12$); OX-$NaHCO_3$ in 42% of high risk vs 17% non-high risk ($P = 0.01$); Table S1], largely due to an association between pleuropulmonary infections, caused mainly by isolates belonging to ST 93 (29), and the responsive phenotype (of such infections 9/12 were CFZ-$NaHCO_3$- and 5/11 were OX-$NaHCO_3$-responsive). Among isolates collected from participants with infective endocarditis ($n = 11$), only 2/11 and 2/7 were CFZ-$NaHCO_3$- or OX- $NaHCO_3$-responsive, respectively. For participants who were allocated to combination therapy with a β-lactam and had high-risk source of BSI, duration of bacteremia was similar for CFZ-responsive and non-responsive [1.5 days (IQR 1–3) and 1.5 days (IQR 1–5) respectively , $P = 0.72$] and for OX-responsive and non-responsive isolates [1 day (IQR 1–2) and 2 days (IQR 1–4) respectively, $P = 0.68$] (Table S2).

Knowing that the risk of persistent bacteremia is multifactorial, and that the $NaHCO_3$ phenotype could potentially be a contributor, we created a multiple regression model. There was no association between $NaHCO_3$-responsive vs non-responsive MRSA strains and persistent bacteremia (Table S3).

Rates of 30- and 90-day mortality were not significantly different among participants who were allocated to combination therapy with a β-lactam and had BSI caused by CFZ- and OX-responsive vs non-responsive strains (Table S2).

DISCUSSION

In this study of 117 MRSA bloodstream isolates from the CAMERA2 trial, the CFZ-$NaHCO_3$- and OX-$NaHCO_3$-responsive phenotype was present in 31% and 25% of isolates, respectively. Among this unique cohort of patients with MRSA bloodstream

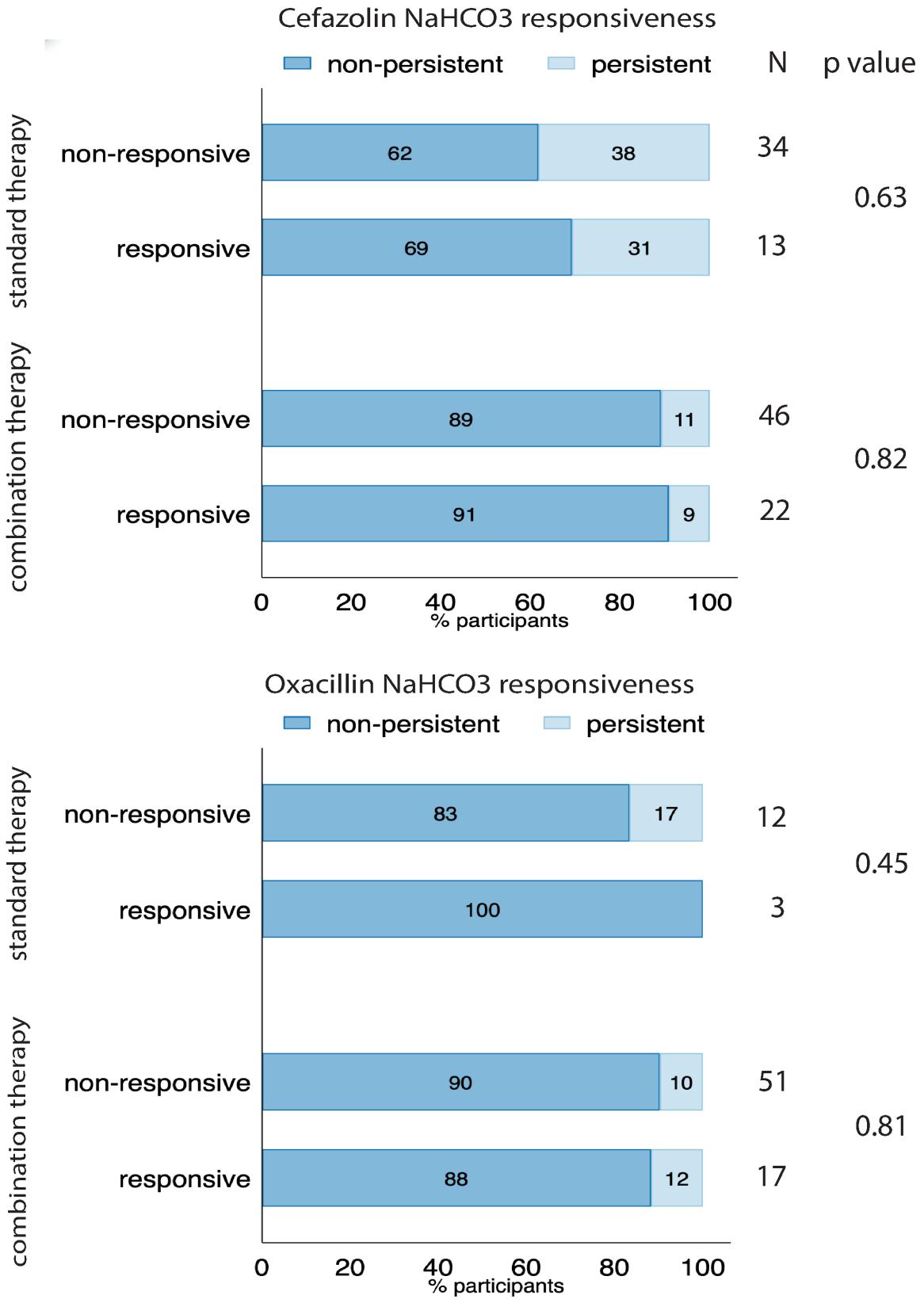

FIG 5 Clinical correlates of the $NaHCO_3$ phenotype. (Top) Rate of persistent bacteremia on day 5 among participants who were allocated to standard or combination therapy and had infections caused by cefazolin-$NaHCO_3$-responsive compared to non-responsive strains. (Bottom) Rate of persistent bacteremia on day 5 among participants who were allocated to standard or combination therapy and had infections caused by oxacillin-$NaHCO_3$-responsive compared to non-responsive strains.

infections who were allocated to treatment with β-lactams, we found no association between persistence of bacteremia at trial day 5 and *in vitro* $NaHCO_3$ responsiveness for either β-lactam. Our laboratory experiments extend previous findings in determining genotypic associations of $NaHCO_3$ responsiveness with the Australian ST 93 lineage and with the presence of SCC*mec IVa* and either a −7G→T or −38A→G SNP in the regulatory region of *mecA*.

The CAMERA2 trial cohort, featuring a subset of patients with MRSA bloodstream infections treated with a β-lactam antibiotic, offered an ideal setting to determine whether the treatment response differs according to the $NaHCO_3$ responsiveness of the infecting *S. aureus*. We used clearance of persistent MRSA bacteremia on trial day 5 as a surrogate marker. While recent studies have shown that even 1 day of persistent positive blood cultures can be associated with higher mortality (30), most authors define persistent bacteremia as positive blood cultures after 5–7 days of active treatment (31). We chose the threshold of 5 days because CAMERA2 showed a positive effect of combination therapy with regard to ongoing bacteremia at this timepoint. Among 70 patients allocated to a β-lactam regimen, we found no association between $NaHCO_3$ responsiveness and persistent bacteremia at day 5. Persistent bacteremia was uncommon for those treated with β-lactams (11%, 19/166). In the subset of participants with isolates tested for $NaHCO_3$ responsiveness, the rate was similar between those with and without $NaHCO_3$ responsiveness.

Our findings differ from previous studies which suggested that the $NaHCO_3$-responsive phenotype was predictive of treatment response to CFZ and OX in rabbit infective endocarditis (IE) models (9) and in a simulated *ex vivo* endocardial vegetation model (11). Our cohort included only 11 participants with infective endocarditis and only 7 of them were allocated to receive a β-lactam, which precluded further analysis of this interesting group of patients. Although it would be unlikely that a single bacterial factor would significantly influence patients' outcomes, the rate of persistent bacteremia was nearly identical regardless of the $NaHCO_3$ responsiveness status. This speaks to the complex and multifactorial nature of determinants important in clearing persistent MRSA bacteremias in humans with multiple background conditions (e.g., diabetes) that likely impact treatment outcomes vs normal rabbits with experimental IE.

Our results suggest that $NaHCO_3$ responsiveness in isolation does not explain the observation that persistent bacteremia was reduced in patients receiving β-lactam therapy. Other mechanistic explanations for the more rapid clearance of bacteremia among those receiving β-lactam therapy may include (i) synergy between β-lactams and host defense peptides (HDPs) such as LL-37. While we did not directly test for such synergy, we did find an association between $NaHCO_3$ responsiveness and susceptibility to daptomycin. Daptomycin and HDPs are both cationic molecules and resistance to daptomycin has been found to correlate with resistance to HDPs (32). Furthermore, Ersoy et al. demonstrated that $NaHCO_3$-responsive isolates were more susceptible to HDP killing and there was additional synergy for killing when β-lactams were combined with HDPs (9). These phenomena were not present in $NaHCO_3$-non-responsive isolates. However, in our current data set, we did not observe faster clearance of bacteremia among patients with $NaHCO_3$-responsive isolates. (ii) Synergy between vancomycin and β-lactams. Such synergy has been well described (5) and several studies have demonstrated a "see-saw effect" where increasing MICs to vancomycin (6) or daptomycin (7) are associated with reduced MICs to β-lactams. We did not directly test for synergy. The limited range of MICs to vancomycin (113/117 isolates with vancomycin MIC <2 mg/L) and daptomycin (113/117 isolates with daptomycin MIC <1 mg/L) precluded demonstration of any "see-saw effect."

We found discordant CFZ- and OX-$NaHCO_3$ response in 24% of isolates. These were most commonly seen in ST 93 and ST 22 where eight and four isolates were CFZ-responsive/OX-non-responsive, and in ST 45 where four isolates were CFZ-non-responsive/OX-responsive. Although such discrepancies between the CFZ-$NaHCO_3$- and OX-$NaHCO_3$-responsiveness assays have been reported before, Ersoy et al. (21) found

the OX-responsive phenotype to be a good proxy for the CFZ-responsive phenotype, wherein 95% of OX-responsive strains were also CFZ-responsive. Further investigation of these discrepancies may be warranted. We note that discordant susceptibility testing has also been described with oxacillin and cefoxitin for MRSA screening (33).

We also investigated for associations between the $NaHCO_3$-responsive phenotype and specific genotypic markers including the sequence types, *mecA* SNPs, and haplotypes. There were significant differences in the rates of $NaHCO_3$ responsiveness according to underlying genetic background of isolates. We found that ST 93, an Australian community-associated MRSA lineage, was more likely to be $NaHCO_3$-responsive than other genetic backgrounds. Our cohort provides a broader geographic and genotypic selection of isolates than previous studies, thus, both confirming and extending previous findings that certain genotypes such as USA300 are more commonly $NaHCO_3$-responsive than comparison genotypes (21). The frequency of $NaHCO_3$ responsiveness among the CAMERA2 isolates was significantly lower than that seen among US isolates, especially for CFZ(21); this may speak to the striking differences in prevalent clonotypes circulating in the USA vs the CAMERA2 cohort, and perhaps to the distinct genotypes found in these latter strains (e.g., *mecA* haplotypes; see below).

As alluded to above, two SNPs in the *mecA* regulatory region were associated with the $NaHCO_3$-responsive phenotype. This finding joins other reports that have shed light on the effect of mutations in the promoter regions of PBPs on the susceptibility phenotype to β-lactams in enterococci (34–36) and MRSA (14, 37). The G-to-T substitution at position −7 in the ribosome binding site (−7G→T) had been previously reported to correlate with several β-lactam hyper-susceptibility phenotypes in MRSA including (i) the $NaHCO_3$-responsive phenotype (13); (ii) a lower OX MIC profile in general (38); (iii) specifically the OX-susceptible MRSA phenotype (OS-MRSA) (39, 40); and (iv) the β-lactam-β-lactamase-susceptible phenotype (13, 14, 41). This SNP had been shown to correlate with lower expression of the *mecA* gene than the wild-type −7G as measured by qRT-PCR. On the other hand, OS-MRSA isolates harboring this SNP that have been exposed to OX revert to a "resistant phenotype" while still harboring this SNP (39, 40), suggesting that other factors are affecting the resistance level to β-lactams. Moreover, a $NaHCO_3$-non-responsive isolate, which originally harbored a −7G allele of the *mecA* gene, remained $NaHCO_3$-non-responsive even after introduction of the −7G→T SNP (20), hinting that the genetic background of the isolate has a greater effect on the expression of this phenotype than any single SNP.

The second SNP in the regulatory region of the *mecA* gene that correlated with the $NaHCO_3$-responsive phenotype is an A-to-G substitution located at position −38 upstream (−38A→G). This SNP has been associated with relatively high OX MICs (40, 42) and a β-lactam-β-lactamase-susceptible phenotype (41). In our study, we found 13 isolates that harbored this SNP which could be further classified into two distinct haplotypes and matching phenotypes. Ten isolates harbored three SNPs in the *mecA* regulatory region, the −38A→G together with a −33C→T and a −7G→T SNPs, and were designated haplotype S 5. These isolates all belonged to ST 93, had a medium range of CFZ MICs (median MIC 64 mg/L) and OX (median MIC 16 mg/L), and 80% (8/10) and 71% (5/7) were CFZ-$NaHCO_3$- and OX-$NaHCO_3$-responsive, respectively. The −33C→T SNP is located in the "−10 box" of the promoter which is the binding site for the *mecA* repressors *blaI* and *mecI*, and its presence was previously correlated with negligible expression of the *mecA* gene (14). The three other isolates harboring the −38A→G SNP also harbored a −33C→T SNP and the WT −7G, and were designated genotype R3. They belonged to ST 5, had higher CFZ MICs (median MIC 256 mg/L) and OX MICs (median MIC 256 mg/L), and were all $NaHCO_3$-non-responsive to both CFZ and OX. Again, it is unclear whether there is a causative effect on the resistant phenotype or whether it is merely an association related to the genetic background.

Our study has several strengths and limitations. It is the first study evaluating a possible clinical effect of the $NaHCO_3$-responsive phenotype on the outcomes to β-lactam treatment in persistent MRSA bacteremia. This is also the largest and

most geographically diverse cohort of MRSA isolates in which the $NaHCO_3$-responsive phenotype had been assessed to date. Nonetheless, the sample size was still limited in power to find a potential association with clinical outcomes (type II error). Our assessment was restricted to the four most common sequence types in the cohort, and findings may not extrapolate beyond these sequence types. The genomic analysis was limited to candidate genes/regions/markers that were previously linked to β-lactam susceptibility in MRSA. An unbiased approach (e.g., genome-wide association study) might reveal new genetic loci; however, a large data set would be needed to achieve genome-wide significance (22).

In conclusion, the β-lactam-$NaHCO_3$-responsive phenotype was associated with several specific genotypic markers but was not associated with lower rates of persistent bacteremia among patients treated with β-lactams.

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Neta Petersiel, Data curation, Formal analysis, Investigation, Writing – original draft | Stefano Giulieri, Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Visualization, Writing – review and editing | Diane S. Daniel, Conceptualization, Investigation, Methodology, Supervision | Sook-Ha Fan, Conceptualization, Formal analysis, Investigation, Methodology | Selvi C. Ersoy, Conceptualization, Formal analysis, Investigation, Methodology | Joshua S. Davis, Conceptualization, Supervision, Writing – review and editing | Arnold S. Bayer, Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review and editing | Benjamin P. Howden, Conceptualization, Supervision, Writing – review and editing | Steven Y. C. Tong, Conceptualization, Formal analysis, Methodology, Supervision, Writing – review and editing.

ETHICS APPROVAL

Because this study was a secondary analysis of previously collected data, reconsent of participants was not required. As part of the initial trial (15), participants provided written informed consent.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (AAC00218-24-s0001.docx). Tables S1 to S4.

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