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Evaluating the potential of novel antimicrobial strategies in a multimodal treatment model for implant-related infections: antibiotics, antimicrobial peptides, bacteriophages, induction heating

Verheul, M.

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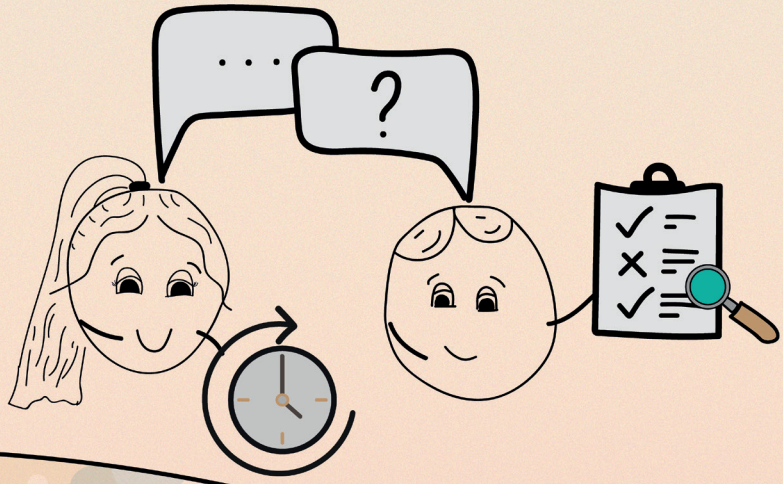

Summarizing discussion

Mariëlle Verheul[1,2]

[1] Leiden University Center for Infectious Diseases (LUCID), Leiden University Medical Center, Leiden, the Netherlands

[2] Department of Orthopedics, Leiden University Medical Center, Leiden, the Netherlands

In this thesis, the effects of phages, non-contact induction heating (NCIH), the antimicrobial peptide SAAP-148, and antibiotics as single- and multimodality approaches on biofilm-associated *S. aureus* on metal implant mimics *in vitro* are described. **Chapter 1** reports the challenges associated with treating biofilm-related orthopedic infections and provides the background of the alternative approaches tested in this thesis. In **Chapter 2**, the effects of phage ISP against *S. aureus* in the various phases of its biofilm cycle are described. The functional and proteomic differences between phage-tolerant bacteria dispersed from biofilms and phage-sensitive bacteria are investigated in **Chapter 3**. The accuracy of predicting phage-antibiotic combinations (PAC) on biofilm-encased *S. aureus* utilizing planktonic counterparts is determined in **Chapter 4**. Further, in **Chapter 5** the effects of NCIH and its combination with SAAP-148 are investigated on biofilms enriched for persisters. Lastly, in **Chapter 6** it is elucidated whether sequential combinations of antibiotics, SAAP-148, or phage ISP with NCIH can enhance NCIH's efficacy in eradicating biofilm-embedded *S. aureus*, allowing the use of lower NCIH temperatures. The findings illustrate that multimodality approaches, i.e., phages combined with antibiotics, NCIH with SAAP-148, or NCIH with antibiotics, are more effective at reducing bacterial biofilms than these approaches alone.

Efficacy of phage monotherapy on *S. aureus* in biofilms and beyond

Although some success has been reported with phage-only treatment, interpreting the effectiveness of phages as single agents against biofilm-associated infections remains complex due to a lack of standardized clinical trials, with subsequent inconsistent results. Furthermore, the large heterogeneity between both biofilm-associated infections and patients with these infections underscores even more the complexity of interpreting the outcomes of non-standardized studies. Hence, standardized testing the effects of phages against bacteria within relevant biofilm models *in vitro* is crucial. Using our *in vitro* biofilm models – which have been refined to mimic the various phases of the biofilm cycle on orthopedic implants (**Chapter 2**) – we found that phage ISP could not effectively eradicate adhered *S. aureus* at the implant surface (**Chapters 2 and 3**). Further, (repeated) exposure of *S. aureus* biofilms to phage ISP demonstrated limited efficacy, resulting in high bacterial loads remaining on the implant (**Chapters 2 and 4**). In agreement, a single dose of phage ISP was ineffective at eliminating *S. aureus* colonized at porcine or human skin models (1). Nevertheless, some *in vitro* studies reported that phages vB_SauM-A and vB_SauM-D reduced the bacterial load and biomass of multi-drug resistant (MDR) *S. aureus* biofilms, and were even more effective than antibiotics (2). In addition, phage Sb-1 reduced the extracellular polysaccharide matrix and bacterial burden of *S. aureus* biofilms (3). In line with our observations, none of the reported *in vitro* studies has shown eradication of bacterial biofilms upon exposure to phage therapy only. We found that the biofilm architecture hampered ISP's efficacy in eradicating biofilms despite the phage's ability to penetrate (im)mature *S. aureus* biofilms and to interact with the bacteria therein (**Chapter 2**).

The efficacy of phages was not only impaired by the biofilm structure but also by the emergence of phage-tolerant bacterial populations. These findings support previous suggestions that the poor efficacy of phages against bacterial biofilms is due to the biofilm matrix and their preference to infect bacteria with less maturity (4). Moreover, a fraction of bacteria dispersed from biofilms survived exposure to high doses of phages and were even able to proliferate in their presence (**Chapter 3**). Our findings showed similar characteristics between biofilm-embedded bacteria and bacteria dispersed from biofilms. Possibly, the

bacterial fraction(s) that survived phage exposure within and dispersed from biofilms exhibit similar phenotypes.

The limited *in vitro* efficacy of phages against biofilm-associated bacteria indicates that phage monotherapy should not be advised in the treatment of biofilm-related infections. Phage therapy should be potentiated, for instance by combining them with other antibacterial approaches (**Chapters 4 and 6**), which will be discussed later. Furthermore, directed evolution of phages could enhance their efficacy and reduce the development of phage-resistant or phage-tolerant bacterial mutants. Phage training on biofilms involves multiple rounds of phage exposure, resulting in the evolution of phages capable of infecting a broader range of bacterial hosts which enhances their ability to eradicate the bacterial load within biofilms (5, 6). Despite the latter, evolved phages may still fail to completely eradicate biofilms, because the heterogeneity among biofilm-encased bacteria facilitates the adaptation and persistence of certain subpopulations. Besides, phage adaptation is a personalized and time-consuming process that may complicate its implementation in clinical practice, as time is very limited in patients suffering from these life-threatening infections. Another theoretical option to increase the efficacy of phages is genetic modification by introducing biofilm matrix-degrading enzymes and/or enzymes that impair quorum sensing, hampering bacterial virulence and dispersion (7, 8). The clinical implementation of such engineered phages, however, is even more complex due to regulatory hurdles and safety concerns.

Since phages are mainly effective against bacteria in planktonic phase, as also demonstrated in this thesis (**Chapters 2, 3, and 4**), they have been utilized to prevent implant-related infections. Su et al. demonstrated that bioceramic scaffolds loaded with a phage cocktail reduced *S. aureus* biofilm formation (9). Another study showed that phages loaded into calcium phosphate powder inhibited biofilm formation and disrupted established *S. aureus* biofilms on the implant surface (10). Notably, the specificity of phages requires identification and characterization of the bacterial species that causes the infection. A possible way to avoid these lengthy and laborious adaptation processes is to use phage cocktails, which have been developed to target the species most commonly observed in these infections and have broad host ranges. However, such cocktails may not be effective against the infecting bacterial strain(s).

Efficacy of non-contact induction heating (NCIH) on *S. aureus* in biofilms

In contrast to phage ISP, applying heat to biofilms with our custom-built induction heating device was highly effective at reducing the bacterial load (**Chapters 5 and 6**). That is, exposure to NCIH at 70 °C and 80 °C was more effective at reducing biofilm-embedded *S. aureus* than exposure to high doses of antibiotics. Importantly, NCIH-induced bacterial killing was observed in the various layers of mature biofilms, indicating effective heat distribution throughout the biofilm (**Chapter 6**).

Previous studies have mostly focused on the effects of NCIH on 24 – 48 hour biofilms, whereas biofilms cultured for at least five days are believed to better represent the maturity of clinical biofilms (11). Interestingly, NCIH was only slightly more effective against bacteria residing within 24 – 48 hour biofilms than in seven-day mature biofilms (**Chapter 5**) (12), indicating that NCIH is effective independent of the biofilm's maturity state. Nevertheless, no studies have reported complete bacterial eradication of *S. aureus* biofilms upon exposure

to NCIH.

The efficacy of NCIH on biofilms *in vitro* varies across different studies. For instance, García-Galán et al. showed that NCIH reduced 24-hour *S. aureus* biofilms by 0.5-log CFU/mL, which is not considered effective given the high bacterial load remaining on the implant surface (13). Other studies, however, reported reductions of at least 2-log CFU/mL upon NCIH exposure, similar to our observations (**Chapters 5 and 6**) (12, 14). The discrepancy in efficacy among these studies may be attributed to differences in bacterial strains and species, the design of the induction coil to uniformly heat the implant, the shape of the implant mimics, and the duration of NCIH exposure. So far, no differences in the efficacy of NCIH have been observed for biofilms on different biomaterials (9).

Further, using temperatures that effectively kill biofilm-embedded bacteria while minimally damaging the peri-implant tissue is pivotal. Recent studies have observed minimal tissue damage as a result of NCIH exposure. That is, exposure to 70 °C maximally induced 517 µm of bone necrosis around biofilm-infected screws, and another study reported tissue damage of less than 1 mm upon exposure to 75 °C (15, 16). Further, thermal damage up to 3 mm was observed in the peri-implant tissue after heating *S. aureus*-infected screws in sheep tibia to 70 or 80 °C. In addition, inflammation and necrosis of soft tissue, but not bone tissue, were reduced by this approach compared with the positive control (17). The peri-implant tissue revealed enhanced neutrophil infiltration after NCIH, which might further potentiate bacterial eradication (16). Still, the influence of NCIH on local tissue inflammation and on the bacteria within the peri-implant tissue remains to be evaluated.

The effects of the antimicrobial peptide SAAP-148 on *S. aureus* biofilms

Various studies have aimed to overcome the limitations of antimicrobial peptides (described in **Chapter 1**) as a treatment approach by reducing their cytotoxicity and increasing their stability through delivery systems. Interestingly, SAAP-148 encapsulated in nanogels and nanoparticles demonstrated lower cytotoxicity compared to the free peptide, while remaining effective against biofilm-embedded *S. aureus* (18, 19). Future studies should elucidate whether formulated SAAP-148 serves as a potent adjuvant for the treatment of implant-related infections and remains effective in eradicating persisters (20) (**Chapter 5**).

Multimodality approaches to eradicate *S. aureus* biofilms

This thesis has demonstrated that the various single-treatment modalities are unable to successfully eradicate biofilms due to the biofilm matrix and the presence of tolerant bacterial subpopulations. Therefore, further investigations in this thesis focused on the efficacy of combinations of such treatment modalities (i.e., multimodal treatment) targeting different aspects of biofilms to eradicate bacterial biofilms and prevent bacterial resistance development (**Chapters 4**, **5**, **and 6**).

Multimodality approaches with enhanced efficacy against biofilms

Combinations of phages with ciprofloxacin, flucloxacillin, or high-dose daptomycin in repeated dosing regimens (**Chapter 4**), or NCIH with rifampicin and ciprofloxacin (**Chapter 6**), and NCIH with SAAP-148 (**Chapters 5 and 6**) in sequential treatment regimens resulted in enhanced killing of biofilm-embedded *S. aureus* compared to the single agents alone. Interestingly, these multimodal approaches allowed lower concentrations of antibiotics and SAAP-148, and lower temperatures for NCIH than when given alone.

Phage therapy is often prescribed in combination with standard-of-care antibiotics to enhance the treatment efficacy against difficult-to-treat infections (21). The phage-antibiotic synergy observed in **Chapter 4** aligns with results from other studies showing that biofilm-encased bacteria exhibit enhanced antimicrobial sensitivity when antibiotics are combined with phages (22). In line with this, combined exposure of 48-hour *S. aureus* biofilms to phage K and vancomycin for 72 hours resulted in synergistic reductions (23). A cocktail of antibiotics (daptomycin and ceftaroline) combined with phages (Sb-1 and Intesti13) synergistically reduced methicillin-resistant *S. aureus* (MRSA) within 40-hour biofilms, without the emergence of phage-resistant bacteria, unlike the controls (24). Importantly, we showed that repetitive exposure of biofilms to phage-antibiotic combinations maximally reduced biofilm-embedded bacterial counts, in agreement with previous findings (25, 26).

Furthermore, studies have shown that phages could reverse beta-lactam resistance in MRSA (27). The risk of treatment failure is significant in implant-associated MRSA infections, which often exhibit resistance to multiple antibiotics (28). Enhancing the efficacy of antibiotics with phages would drastically improve treatment outcomes for patients with multidrug-resistant (MDR) bacterial infections and help curb antimicrobial resistance (AMR). Besides, exposure of biofilm-dispersed *S. aureus* to ISP in the presence of suboptimal doses of vancomycin or flucloxacillin reduced the emergence of phage-tolerant bacteria compared to exposure to ISP alone (**Chapter 3**). Thus, combined phage-antibiotic treatment regimens may also prevent the rise of phage-tolerant and potentially phage-resistant bacteria.

Exposing bacterial biofilms to phages before antibiotics, but not vice versa, can increase treatment efficacy (29, 30). For instance, exposure of biofilms to phages prior to antibiotics enhanced the antibiotic efficacy at low concentrations and prevented the emergence of resistant mutants (29). However, this thesis did not investigate the effects of such sequential exposures due to hurdles of implementing this sequence in clinical practice. Such a phage-first approach would involve discontinuing standard-of-care antibiotics, which is neither feasible nor recommended in many clinical situations.

Our findings demonstrated that sequential combinations of SAAP-148 or antibiotics with NCIH synergistically reduced biofilm-encased *S. aureus* (**Chapter 6**). Further, the combination of SAAP-148 followed by NCIH at 60 °C was somewhat more effective at reducing persister-enriched biofilms than the single approaches alone (**Chapter 5**). Hence, antimicrobial peptides may be a promising additive to the treatment of implant-associated infections. Although we were the first to describe the effects of NCIH combined with antimicrobial peptides, others have also shown that NCIH combined with antibiotics was more effective against biofilms than either approach alone (12, 14, 16).

Interestingly, NCIH combined with rifampicin and ciprofloxacin, for which the MRSA strain is resistant, resulted in enhanced efficacy of these antibiotics (**Chapter 6**). Previous research suggested that NCIH may target porins or efflux pumps in the cell membrane that are involved in antibiotic resistance (12). In addition, we observed that NCIH was more effective at reducing MRSA biofilms than methicillin-sensitive *S. aureus* (MSSA), suggesting that antibiotic resistance may entail trade-offs that increase the bacterial sensitivity to heat exposure. NCIH offers a promising approach to eliminate MDR bacterial biofilms by increasing their sensitivity to antimicrobial treatment regimens.

In addition, NCIH can be used in a drug-release system for antimicrobials loaded in magnetic nanoparticles, coatings, or hydrogels, ensuring controlled and targeted drug delivery at the

site of infection to eradicate biofilm-embedded bacteria. For instance, induction heating of rifampicin-loaded coatings on titanium discs resulted in 3-log reductions of *S. aureus* in biofilms (31).

Multimodality approaches with impaired efficacy against biofilms

In line with previous findings, we observed antagonistic interactions between phage ISP and rifampicin or gentamicin (**Chapter 4**) (32-34). The findings in **Chapter 4** clearly demonstrate that screening phage-antibiotic combinations on planktonic bacteria can predict the occurrence of antagonistic interactions. Planktonic testing thereby provides a valuable first-line screening method for selecting phage-antibiotic combinations to treat biofilm-associated infections.

In addition, we observed that NCIH exposure of biofilms reduced the efficacy of sequential exposure to phage ISP (**Chapter 6**). Previous studies showed that NCIH reduced the density of the biofilm matrix, which may enhance the penetration of antimicrobials into the biofilm (12). Moreover, NCIH may affect the integrity of the bacterial cell membrane and thereby enhance the uptake and efficacy of antimicrobials. Earlier studies indicated that hyperthermia enhanced the metabolic activity and uptake of subsequently administered vancomycin by *S. aureus* in biofilms (35, 36). Hence, we hypothesized that NCIH could potentiate the efficacy of phages by enhancing their biofilm penetration and increasing their replication within bacteria. However, combining phages with NCIH reduced the efficacy of ISP (**Chapter 6**). Although this suggests that NCIH altered the cell wall integrity or bacterial metabolism, thereby hampering phage adsorption, injection, and/or replication, this remains to be elucidated.

Elimination of tolerant bacterial populations

Bacterial tolerance to antimicrobial strategies is the major challenge in the treatment of biofilm-related infections. These tolerant bacteria often exhibit reduced metabolic activity and reduced target activity, thereby hampering the efficacy of antimicrobials (37). Persisters are tolerant bacteria within a population in which the bulk of the population is sensitive to antimicrobial therapy (38). These persisters are particularly known to cause relapse of infection when antibiotic treatment is discontinued (39). We showed that phage ISP was ineffective against persister-enriched biofilms (**Chapter 2**) and that a subpopulation of bacteria dispersed from biofilms tolerated high-dose phage exposure (**Chapter 3**). Notably, exposure to NCIH at 70 °C eradicated the complete load of persisters within biofilms, and exposure to SAAP-148 slightly potentiated the efficacy of NCIH at 60 °C (**Chapter 5**).

A variety of persister-inducing models have been used in literature to investigate the efficacy of antimicrobial therapies on persisters. Importantly, the type of exposure used to induce persisters affects the metabolic profile of the persister population and, thereby, their response to treatment regimens (40, 41). The use of different persister-inducing models may explain the contrasting findings regarding the phages' efficacy against persisters: successful in Tkhilaishvili et al. (3), but ineffective in **Chapter 2**.

Bacteria within persister-enriched biofilms proliferated in the presence of ISP (**Chapter 2**), suggesting that mechanisms other than metabolic dormancy underlie the phage-tolerance of these bacteria. Interestingly, proteome analysis of phage-tolerant bacteria in **Chapter 3** revealed upregulation of proteins previously observed in persisters in other studies. For instance, proteins down-regulated in phage-tolerant bacteria were involved in purine

metabolism and ribosome function (42), whereas proteins that were up-regulated were involved in cell wall organization and two-component regulatory systems (41). It remains to be elucidated whether the phage-tolerant bacteria in the biofilm-dispersed fraction (**Chapter 3**) and the antibiotics-induced persisters in biofilms (**Chapter 2**) display similar phenotypes that contribute to their ability to survive the effects of phages.

Future research

***In vitro* biofilm model**

The biofilm models used in this thesis have been developed to represent clinically relevant biofilms on metal implants to mimic an implant-associated infection. These biofilms were cultured for seven days without refreshing the culture medium; longer incubation times would further deprive the bacteria of nutrients. Nevertheless, our *in vitro* biofilms may be more challenging to eradicate than those in the clinic due to enhanced nutrient deprivation, the absence of shear stress, and the lack of immune cells and factors.

Therefore, future studies should refine these *in vitro* biofilm models to better mimic the biofilm composition, complexity, and antimicrobial response observed *in vivo*. Utilizing systems such as the recently developed "Bio-Rocker" platform, which allows biofilm culturing in plates while controlling for shear stress and temperature (43), and 3D models that incorporate titanium implants, peri-implant tissue, and biofilms (44), would significantly enhance the clinical relevance of these models. Implant-related infections on a chip would be the ultimate tool for investigating the dynamics between bacteria and various host factors, including blood plasma, synovial fluid, immune cells, temperature, pH, oxygen supply, the tissue-biomaterial interface, and physiological fluid flow. The latter influences both nutrient availability and the dynamics of biofilm dispersal. De Bleeckere et al. recently developed a synthetic synovial fluid model to investigate biofilm formation and its response to antibiotics *in vitro*, an important step towards mimicking the physiological microenvironment (45). Surely, such optimized models should be compared with *in vivo* biofilms regarding biofilm thickness, composition, dynamics, and functional responses under antibiotic treatment, as well as the (combined) alternative approaches tested in this thesis.

In these advanced biofilm models, research should focus more on bacteria dispersed from biofilms. This bacterial population is highly relevant in the clinic and must be eradicated to reduce the risk of infections elsewhere, which may be even more antimicrobial tolerant. Currently, little is known about the *in vivo* phenotypes of biofilm-dispersed bacteria and their responses to antimicrobial treatment. Future studies should determine whether phage-tolerant bacteria (**Chapters 2 and 3**) similarly arise in more sophisticated biofilm models and *in vivo*, and investigate strategies to eradicate them.

Clinical aspects of alternative treatment modalities

Antibiotic concentrations used in *in vitro* assays are often based on blood serum concentrations that can be maximally reached in patients. However, it should be noted that the penetration of these antibiotics into the peri-implant tissues, particularly bone, is often reduced compared to serum levels due to limited vascularization (46, 47). Future studies should focus on defining the pharmacokinetics and pharmacodynamics (PK/PD) of antibiotics, and the (combined) alternative modalities tested here, to select safe and effective administration and dosing regimens in these patients.

Moreover, the biofilm presents another challenge by impeding antibiotic diffusion to bacteria within the biofilm. The binding of antimicrobials to biofilm matrix components can further decrease the free antimicrobial concentrations that are available to target these bacteria (48). Although the enhanced efficacy of repeated dosing of phage-antibiotic combinations (**Chapter 4**) and the sequential exposure of biofilms to NCIH and antibiotics or SAAP-148 (**Chapter 6**) may suggest that these combined approaches increase the free concentration of antibiotics and SAAP-148 within the biofilm, further research is needed to confirm this.

To better predict the effects of combined treatment on biofilms using planktonic bacteria (**Chapter 4**), the antimicrobials' mechanisms of action and free biofilm-embedded concentrations should be deciphered in greater detail. Accordingly, machine-learning models can be used to predict the treatment efficacy of the multimodal approaches based on mechanistic interactions and actual concentrations. Utilizing *in vitro* checkerboard assays will help improve the accuracy of these prediction models. Ultimately, the goal is to identify which treatment combinations are most effective *in vivo* based on *in vitro* findings. This, however, requires highly sophisticated systems and models to understand the PK/PD and efficacy of these treatment modalities and their combinations.

The effect of local (i.e., intra-articular, intra-osseous) delivery of antibiotics is advocated by some with the argument to achieve higher local concentrations (49), for instance when attached to a calcium phosphate vector as an adjunct to intravenous prophylaxis (50, 51). Some small case studies claimed that two-stage revision surgery with intra-articular irrigation, as well as local continuous antibiotic perfusion concomitant to DAIR, enhanced implant survival rates in PJI (52, 53). However, the effectiveness of local antibiotic delivery as a treatment option remains debatable in the absence of randomized clinical trials.

Surgical debridement of infected and necrotic tissue is most probable key as part of a multimodal treatment approach. The intraoperative challenge is to treat an infected joint as a tumor, but without resecting vital non-infected and non-necrotic tissue. Hence, the development of intraoperative bio-imaging techniques to visualize infected tissue and bacteria will improve the multimodal treatment efficacy and the patient's remaining functionality (54-56). For that matter, implant-related infections can spread to the bone, invade, and persist within osteoblasts, osteoclasts, osteocytes, and macrophages, leading to osteomyelitis (57). Although this thesis did not cover this complication, future research should investigate the extent to which these multimodal approaches can target these persisting intracellular bacteria. Induction heating would not be suitable for targeting these infected bone cells, as it only heats metals. However, antimicrobial peptides, phages, antibiotics, and their combinations – formulated to enhance their uptake and intracellular efficacy – may offer an interesting strategy for targeting these intracellular bacterial reservoirs.

Influence of multimodality approaches on host (immune) cells

The host immune system plays a crucial role in (resolving) these infections. *S. aureus* biofilms notoriously recruit myeloid-derived suppressor cells (MDSCs), which reduce proinflammatory antimicrobial responses and allow the infection to persist (58). Notably, linking polyethylene glycol (PEG) groups to the C-terminus of SAAP-148 previously enhanced granulocyte chemotaxis and skewed macrophage differentiation toward a proinflammatory phenotype (59). We have also observed that phage ISP induces granulocyte migration *in vitro* at multiplicities of infection ranging from 100 to 1000 (unpublished data). Yet, it remains unclear whether such high phage-granulocyte ratios are realistic in a clinical setting

and how ISP influences immune effector functions. Aside from the observation that NCIH exposure enhanced neutrophil infiltration in the peri-implant tissue (16), its effect on the local immune cell response remains elusive. Future research should thoroughly evaluate the immune-modulatory properties of these approaches and their combinations, using clinically relevant models that mimic the local conditions in these infections, including MDSCs.

Conclusions

The eradication of implant-related biofilm infections requires multimodal strategies, of which adequate surgical resection of infection tissue is a key component. Importantly, the correct combinations of NCIH, antimicrobial peptides, antibiotics, and phages were more effective in reducing biofilm-embedded *S. aureus* on a metal implant mimic *in vitro* than the single treatments. Successful treatment combinations preferably target different mechanisms to reduce the emergence of bacterial tolerance and resistance. Research should not only focus on the bulk of the biofilm, but also on the (development of) tolerant bacterial populations that may cause treatment failure and can cause infections elsewhere via dispersion. Furthermore, future studies should address the mechanisms of action underlying the enhanced treatment efficacy of successful multimodality approaches. Additionally, *in vitro* biofilm models should be refined to mimic the host's physiological microenvironment to investigate the efficacy and immunomodulatory properties of these strategies. Incorporating these factors in PK/PD and efficacy prediction models will guide appropriate treatment selection and thereby improve treatment outcomes for patients with these infections.

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