Adjunctive transferrin to reduce the emergence of antibiotic resistance in Gram-negative bacteria

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Background: New strategies are needed to slow the emergence of antibiotic resistance among bacterial pathogens. In particular, society is experiencing a crisis of antibiotic-resistant infections caused by Gram-negative bacterial pathogens and novel therapeutics are desperately needed to combat such diseases. Acquisition of iron from the host is a nearly universal requirement for microbial pathogens—including Gram-negative bacteria—to cause infection. We have previously reported that apo-transferrin (lacking iron) can inhibit the growth of Staphylococcus aureus in culture and diminish emergence of resistance to rifampicin.

Objectives: To define the potential of apo-transferrin to inhibit in vitro growth of Klebsiella pneumoniae and Acinetobacter baumannii, key Gram-negative pathogens, and to reduce emergence of resistance to antibiotics.

Methods: The efficacy of apo-transferrin alone or in combination with meropenem or ciprofloxacin against K. pneumoniae and A. baumannii clinical isolates was tested by MIC assay, time–kill assay and assays for the selection of resistant mutants.

Results: We confirmed that apo-transferrin had detectable MICs for all strains tested of both pathogens. Apo-transferrin mediated an additive antimicrobial effect for both antibiotics against multiple strains in time–kill assays. Finally, adding apo-transferrin to ciprofloxacin or meropenem reduced the emergence of resistant mutants during 20 day serial passaging of both species.

Conclusions: These results suggest that apo-transferrin may have promise to suppress the emergence of antibiotic-resistant mutants when treating infections caused by Gram-negative bacteria.

Introduction

The discovery of antibacterial agents in the 1930s fundamentally transformed medicine.1 Unfortunately, seven decades of medical advances enabled by antibiotics are now seriously threatened by the convergence of relentlessly rising antibiotic resistance and the alarming and ongoing withdrawal of most major pharmaceutical companies from the antibiotic market. While critically needed, any antibiotic that is developed and used clinically will cause selective pressure resulting in resistance, ultimately undermining its effectiveness. Thus, strategies are needed to slow the emergence of antibiotic resistance and prolong the useful lives of effective antibiotics.

Virtually all microbial pathogens require exogenous sources of iron to survive and proliferate, suggesting that approaches targeting iron sequestration hold promise as a novel antimicrobial strategy.2–6 Transferrin is the primary iron-binding protein that governs free iron levels in mammalian blood.6,7 We recently reported that human apo-transferrin had potent cross-kingdom
antimicrobial activity, inhibiting the growth of *Staphylococcus aureus*, *Acinetobacter baumannii* and *Candida albicans*. Apo-transferrin synergized with rifampicin in vitro against *S. aureus*, decreasing the rifampicin MIC from 0.15 to 0.019 mg/L—an 8-fold decrease. Moreover, apo-transferrin markedly diminished the emergence of resistance to rifampicin in *S. aureus*. Given the breadth of apo-transferrin activity, we sought to determine whether apo-transferrin could also reduce the emergence of antibiotic resistance among Gram-negative bacteria with a high propensity to develop resistance. We focused on *Klebsiella pneumoniae* and *A. baumannii* as key pathogens that have become extremely drug resistant in the clinic. We chose to focus on the antibiotics meropenem and ciprofloxacin as β-lactams and quinolones are the most common classes of antibiotics used to treat nosocomial Gram-negative bacteria. Furthermore, both carbapenems and quinolones are critical agents to preserve (i.e. reduce emergence of resistance) to. Finally, meropenem has a relatively low rate of emergence of resistance while quinolones have relatively high rates, enabling combinatorial testing with antibiotics across a spectrum of rates of resistance emergence.

### Materials and methods

#### Microbial strains

The *A. baumannii* and *K. pneumoniae* clinical isolates utilized in this study are summarized in Table 1.

| Species          | Strain | apo-transferrin (mg/L) | ciprofloxacin (mg/L) | meropenem (mg/L) |
|------------------|--------|------------------------|----------------------|------------------|
| *A. baumannii*   | HUMC1  | 4                       | 256                  | 128              |
|                  | LAC-4  | 8                       | 16                   | 16               |
|                  | AB074  | 4                       | 16 (0.5)             | <0.5             |
|                  | VA-AB21| 2                       | 16 (0.5)             | <0.5             |
|                  | VA-AB48| 2                       | <1 (0.5)             | <0.5             |
|                  | UH267  | 4                       | <1 (0.5)             | <0.5             |
|                  | AB046  | 8                       | 128                  | 2                |
|                  | UH516  | 8                       | >256                 | 256              |
|                  | UH569  | 4                       | 128                  | 128              |
|                  | UH118  | 8                       | 128                  | 64               |
|                  | KPC-KP1| 32                      | 32                   | 32               |
|                  | KPC-KP2| 512                     | 32                   | 64               |
|                  | KPC-KP3| 32 (0.5)                | 32                   | 128              |
|                  | KPC-KP4| 512                     | 32                   | 16               |
|                  | KPC-KP5| 16 (0.5)                | 64                   | 32               |
|                  | KPC-KP6| >512                    | 32                   | 16               |
|                  | KP1    | 128 (0.5)               | 0.0156               | 0.0625           |
|                  | KP3    | 128 (0.5)               | 0.03125              | 0.0625           |
|                  | KP4    | 8                       | 0.03125              | 0.0625           |
|                  | KP5    | 16 (0.5)                | 0.0625               | 0.125            |
|                  | KP6    | 16 (0.5)                | 0.0625               | 0.125            |

### Antimicrobial agents

Apo-transferrin (iron-depleted, Sigma-Aldrich, catalogue number T1147) was made fresh for each experiment. Stock solutions of apo-transferrin were made in OmniTrace Ultra Water (EMD Millipore Corporation, catalogue number WX0003-6).

Ciprofloxacin (Sigma-Aldrich, catalogue number 17850) was freshly prepared for each experiment. Stock solutions of ciprofloxacin (5 mg/mL) were made in 0.05 M HCl prepared with OmniTrace Ultra Water.

Meropenem (APP Pharmaceuticals, catalogue number 350720) was freshly prepared for each experiment. Stock solutions (50 mg/mL) were made in RPMI 1640 medium (Thermo Fisher Scientific, catalogue number 11875085).

### Inoculum preparation

Overnight cultures of the bacteria (*A. baumannii* or *K. pneumoniae*) grown in tryptic soy broth were diluted 1:100 in Mueller–Hinton II (MH2) broth and subcultured until OD600 reached 0.5. Bacteria were collected by centrifugation at 3500 g for 5 min, washing the pellet three times with RPMI 1640 medium. The washed pellet was resuspended in RPMI 1640 medium and diluted to a working concentration of 1× 10⁶ cfu/mL. Bacterial density was confirmed by plating serial dilutions on tryptic soy agar (TSA) plates and extrapolating cfu/plate to cfu/mL.

### Drug susceptibility testing

Antibiotic MIC assays should be done with *A. baumannii* and *K. pneumoniae* in MH2 broth according to CLSI guidelines. However, because the extremely high levels of iron in MH2 broth affect the antimicrobial activity of apo-transferrin, we used our previously described MIC assay in RPMI 1640 medium as it does not have excess iron in the basal formulation.

The inoculum was prepared as described above. MIC assays were conducted in standard 96-well, round-bottom (U-shaped) plates. For single drug susceptibility testing, serial 2-fold dilutions of the drug were made in columns across the plate. Bacteria-alone and medium-only wells were included as positive and negative controls, respectively. Appropriate wells were inoculated by adding 100 μL/well of 1× 10⁶ cfu/mL washed bacteria for a total of 1×10⁵ cfu/well. Plates were incubated for 24 h at 37 °C.

Due to trailing growth noted in early experiments (akin to that found with fungal susceptibility testing) we allowed for some visible pellet in the MIC assays. Antibiotics were added in duplicate rows to make a final concentration of D×, 1/2×, 1/4× and 1× the MIC for each strain. In combination wells, 50 μL of apo-transferrin was added in duplicate rows to make a final concentration of D×, 1/2×, 1/4×, 1/8× and 1× the MIC for each strain. In combination wells, 50 μL of ciprofloxacin or meropenem was added in serial 2-fold dilutions across the columns. Bacteria (100 μL, 10⁶ cfu/mL) were added and the volume of each well was adjusted to a final volume of 200 μL by adding RPMI 1640 medium. Plates were incubated for 24 h at 37 °C.

To evaluate for synergy or antagonism, the fractional inhibitory concentration index (FICI) was calculated using the following equation: FICI = FICA × FICB = (CA/MICA) × (CB/MICB), where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and CA and CB are the concentrations of the drugs in combination, respectively. By convention, synergy was defined as FICI ≤ 0.5, no interaction was defined as FICI > 0.5–4.0 and antagonism was defined as FICI > 4.0.

### Time–kill assays

Time–kill assays allow for a more sensitive readout and include data related to the kinetics of antimicrobial activity, in contrast to the MIC assay.
We used a variation of this assay that utilizes the metabolism of resazurin (Sigma–Aldrich, catalogue number R7017) as a surrogate marker for cfu density, which has previously been used in drug susceptibility assays for both prokaryotic and eukaryotic cells.14–19

The inoculum was prepared as described above. The time–kill assay was set up in 96-well plates with 4–6 technical replicates for each experiment. Edge rows were filled with 200 µL of PBS to minimize test-well evaporation. Antibiotic (ciprofloxacin or meropenem) was added to each well (50 µL/well at 0×, 1/2× or 1× the MIC) with/without apo-transferrin (50 µL/well at 0×, 1/2×, 1× or 3× the MIC). Bacteria-alone and medium-only wells were included as controls. Appropriate wells were inoculated by adding 100 µL/well of 1×10^6 cfu/mL washed bacteria for a total of 1×10^5 cfu/well. Resazurin (0.1% aqueous solution) was added at 20 µL/well and plates were incubated for 24 h at 37°C. Following incubation, fluorescence measurements (544 nm excitation; 590 nm emission) were recorded at 0, 1, 2, 4, 6, 8 and 24 h using the FLUOstar Omega plate reader (BMG LABTECH GmbH, Germany).14 The mean value of the negative control (baseline) was subtracted from the mean value of the technical replicates at each experimental timepoint. Statistical analysis was performed using the Mann–Whitney U-test (α=0.05). An eight-point curve was established to define the range of the linear relationship between cfu and fluorescence signal produced by the metabolism of resazurin. Bacteria were adjusted to an initial concentration of 5×10^5 cfu/mL, followed by 2-fold serial dilutions across the plate. Each dilution had at least six technical replicates. At each timepoint, fluorescence was measured and correlated to cfu density, which was determined by plating serial dilutions.

**Metal-addition reversal of apo-transferrin activity**

Transferrin is known to bind optimally to iron, but is able to bind zinc in a more limited capacity.20–22 Free iron in the blood is scarce (i.e. <10^{-24} M) and most iron that is not already bound by apo-transferrin will be bound in the form of haem.23 To determine the impact of free metals on the inhibition of microbial growth by apo-transferrin, MIC tests were repeated as described below by adding FeCl₃ (Sigma–Aldrich, catalogue number 157740), ZnCl₂ (Sigma–Aldrich, catalogue number 793523) or haemin (Sigma–Aldrich, catalogue number H9039-1G) to saturate apo-transferrin.

The inoculum was prepared as described above. Metal-reversal MIC assays were set up in 96-well plates with three different concentrations of FeCl₃, ZnCl₂ and haemin. Molar equivalents of each metal source were added at 1/10×, 1× and 10× the apo-transferrin MIC. Bacteria (1×10^6 cfu) were added to each well, filling to 200 µL with RPMI 1640 medium. Plates were incubated at 37°C for 24 h.

**Sub-MIC 24 h passage and selection of antibiotic-resistant mutants**

Bacteria were passaged for 24 h in sub-MIC concentrations of ciprofloxacin and meropenem with/without apo-transferrin. Bacteria (10^6 cfu/mL) were passaged in 2 mL of RPMI 1640 medium supplemented with 750 µg/mL apo-transferrin, with/without ciprofloxacin or meropenem at 1/2× the MIC, and incubated at 37°C for 24 h. Apo-transferrin-alone and no-drug control cultures were included as well. Antibiotic-resistant bacteria were quantified by plating cultures on TSA plates with/without 16 mg/L ciprofloxacin or meropenem. While 16 mg/L is above established breakpoints, the goal of these selection experiments was to distinguish major shifts in susceptibility in the presence or absence of transferrin (i.e. changes from highly susceptible to highly resistant).

**Sub-MIC serial 20 day passage and selection of antibiotic-resistant mutants**

Antibiotic-susceptible strains of A. baumannii and K. pneumoniae were passaged for 20 days with sub-MIC concentrations of ciprofloxacin and meropenem with/without apo-transferrin. Control groups with apo-transferrin only or no drug were passaged as well. During the first 5 days of passage, bacteria were grown in antibiotics at 1/4× the MIC and apo-transferrin at 1× the MIC. The cultures were incubated in 24-well plates with 1 mL of RPMI 1640 medium at 37°C without shaking. Daily passages of 10 µL/culture were passaged into fresh RPMI 1640 medium. Antibiotic susceptibility was evaluated by plating bacteria on selection agar containing 2× or 10× the passage concentration of antibiotic 5, 10, 15 and 20 days post-inoculation. Control groups were also plated on ciprofloxacin and meropenem selection agar. Bacterial growth on the selection agar was used to determine the increase in antibiotic concentration to be used for the next five passages. The concentration of apo-transferrin was increased 2× every 5 days. Glycerol stocks of the strains were generated every 5 days. After 20 days, all groups were plated on TSA with/without 16 mg/L ciprofloxacin or meropenem.

**Statistical analysis**

Changes in susceptibility, frequency of resistance emergence and blood bacterial density were compared between groups using the non-parametric Mann–Whitney U-test (α=0.05). Time to moribundity was compared using the non-parametric log-rank test (α=0.05).

**Results**

**Monotherapy MICs**

Clinical isolates of A. baumannii and K. pneumoniae were tested for susceptibility to ciprofloxacin, meropenem and apo-transferrin. The tested strains demonstrated a range of susceptibilities to ciprofloxacin and meropenem (Table 1). There was no correlation between susceptibility to ciprofloxacin or meropenem and susceptibility to apo-transferrin for strains of either A. baumannii or K. pneumoniae. However, there was a much broader range of apo-transferrin susceptibility for the K. pneumoniae strains (8 to 512 mg/L) compared with the A. baumannii strains (2 to 8 mg/L).

After characterizing the susceptibility of 21 A. baumannii and K. pneumoniae strains, we selected representative drug-susceptible and -resistant strains for each species for further investigation (Table 1).

**Combination MICs**

We subsequently tested whether combining apo-transferrin with ciprofloxacin or meropenem had an antagonistic or synergistic effect. Combining the drugs demonstrated additivity, rather than antagonism or synergy for the majority of strains tested, when compared with monotherapy (Table 2). KPC-KP6 was the single exception, with its ciprofloxacin FICI (4.83) slightly crossing the threshold for synergy. This finding could be due to the extreme tolerance of KPC-KP6 to apo-transferrin (MIC >512 mg/L). Conversely, the meropenem and apo-transferrin combination for KPC-KP2 approached a synergistic benefit (FICI=0.66), but did not cross the threshold for synergy (0.5).

**Time–kill assays**

To make time–kill measurements feasible for multiple combination regimens at multiple timepoints, we used resazurin bacterial metabolism as a high-throughput viability assay. We verified that the metabolism of resazurin by viable bacteria produced a reliable
surrogate marker for cfu density by plotting the relationship between the fluorescence signal produced by resazurin and cfu counts. We found a positive correlation between the \textit{A. baumannii} HUMC1 counts and the fluorescence signal, which decreased proportionally as cells entered the stationary phase and became less metabolically active (Figure S1, available as Supplementary data at JAC Online).

Next, we assessed whether apo-transferrin provided an additive benefit in the rate or magnitude of bacterial killing by the antibacterial agents. Using the resazurin assay, we observed drug-drug interactions when apo-transferrin was combined with either ciprofloxacin or meropenem. The apo-transferrin/ciprofloxacin combination resulted in greater killing effects for \textit{A. baumannii} strains HUMC1, UH118, and VA-AB21, while no benefit was observed for AB074 (Table 3, Figure S2 and Figure S3). The apo-transferrin/meropenem combination also produced greater killing effects for \textit{A. baumannii} strains UH118, VA-AB21 and AB074, with no effect on HUMC1 (Table 3, Figure S2 and Figure S3).

\textit{K. pneumoniae} strains have a faster growth rate compared with \textit{A. baumannii} in RPMI 1640 medium. The difference in growth rate made it impractical to use the resazurin assay for \textit{K. pneumoniae}, as the resazurin became oxidized very quickly by metabolic by-products of the rapid growth. Therefore cfu counts were plated to determine the time–kill curves for KPC-KP1. For this particular strain we did not observe an additive benefit of the addition of apo-transferrin at 1x the MIC when added to ciprofloxacin or meropenem at 1x the MIC (Table 3).

### Metal-addition reversal of apo-transferrin activity

We have previously demonstrated that apo-transferrin does not need to be in direct contact with the pathogen to be effective, as its antimicrobial property is mediated by the sequestration of free iron.\(^8\) As a result, we tested whether it is possible to reverse the antimicrobial effect of apo-transferrin by spiking the culture medium with FeCl\(_3\), ZnCl\(_2\) or haemin—each of which was added at 1\,1\% or 10\,1\% the apo-transferrin MIC molar equivalent (Table 4). The presence of ZnCl\(_2\) was unable to effectively reverse the activity of apo-transferrin for seven of the \textit{A. baumannii} and \textit{K. pneumoniae} tested strains, only demonstrating an effect at a 10\,1\% molar equivalent for the \textit{A. baumannii} AB074 and \textit{K. pneumoniae} KPC-KP6 strains. By contrast, FeCl\(_3\) was able to reverse the antimicrobial effect of apo-transferrin against one-fifth of the \textit{A. baumannii} strains and three-quarters of the \textit{K. pneumoniae} strains tested. Surprisingly, haemin was best able to rescue bacteria from apo-transferrin, with all nine strains surviving at 10\,1\%, three at 1\,1\% and two at 1/10\,1\% the molar equivalent of apo-transferrin.

### In vitro selection of antibiotic-resistant bacteria

One of the major advantages of a putative transferrin adjunct therapy is the ability to reduce the emergence of antibiotic resistance. As such, we explored whether apo-transferrin is able to reduce the emergence of resistance to the antibacterial agents in serial passage. Mutants were selected by passaging \textit{A. baumannii} and \textit{K. pneumoniae} strains overnight in sub-MIC concentrations of

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**Table 2. Activity of antibiotics combined with apo-transferrin**

| Species          | Strain     | Resistance | Drug     | FICI (average) | Result       |
|------------------|------------|------------|----------|----------------|--------------|
| \textit{A. baumannii} | HUMC1 | resistant | ciprofloxacin | 2               | no interaction |
|                  | UH118     | resistant | ciprofloxacin | 2               | no interaction |
|                  | LAC-4     | resistant | ciprofloxacin | 2               | no interaction |
|                  | AB074     | susceptible | ciprofloxacin | 2.5             | no interaction |
|                  | VA-AB21   | susceptible | ciprofloxacin | 2               | no interaction |
|                  | HUMC1     | resistant | meropenem   | 2               | no interaction |
|                  | UH118     | resistant | meropenem   | 2               | no interaction |
|                  | LAC-4\(^a\) | susceptible | meropenem   | 2               | no interaction |
|                  | AB074     | susceptible | meropenem   | 2               | no interaction |
|                  | VA-AB21   | susceptible | meropenem   | 2.5             | no interaction |
| \textit{K. pneumoniae} | KPC-KP1 | resistant | ciprofloxacin | 2.5             | no interaction |
|                  | KPC-KP2   | resistant | ciprofloxacin | 2.67            | no interaction |
|                  | KPC-KP6   | resistant | ciprofloxacin | 4.83            | antagonism |
|                  | KP3       | susceptible | ciprofloxacin | 2.5             | no interaction |
|                  | KP4       | susceptible | ciprofloxacin | 2               | no interaction |
|                  | KPC-KP1   | resistant | meropenem   | 2               | no interaction |
|                  | KPC-KP2   | resistant | meropenem   | 0.66            | no interaction |
|                  | KPC-KP6   | resistant | meropenem   | 1.08            | no interaction |
|                  | KP3       | susceptible | meropenem   | 2               | no interaction |
|                  | KP4       | susceptible | meropenem   | 1.75            | no interaction |

Synergy/antagonism was determined for transferrin/ciprofloxacin and transferrin/meropenem combinations using the FICI method.\(^10\) LAC-4 is susceptible to meropenem when cultured in MH2 broth as per CLSI standard protocol; however, it is resistant when cultured in RPMI 1640 medium.

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We found that apo-transferrin had antimicrobial activity against A. baumannii and K. pneumoniae strains. The KPC-KP1 time–kill assay was performed by plating and enumerating cfu instead of the resazurin assay because of differential growth rates between the species. We also selected for resistant mutants by passaging bacteria at 1\%, 10\% or 100\% of the MIC of apo-transferrin. FeCl₃, ZnCl₂ or haemin was supplemented as ZnCl₂ was generally ineffective. Both able to reverse the efficacy of apo-transferrin, where-

### Table 4. Reversal of transferrin activity by metal supplementation

| Species   | Strain | MIC of apo-transferrin (mg/L) | FeCl₃ | ZnCl₂ | Haemin |
|-----------|--------|-------------------------------|-------|-------|--------|
| A. baumannii | HUMC1  | 4                             | −     | −     | +      |
|           | Uh118  | 8                             | −     | −     | +      |
|           | Va-Ab21 | 2                            | ++    | −     | −      |
|           | Ab074  | 4                             | −     | +     | +      |
|           | Lac-4  | 8                             | −     | −     | +      |
| K. pneumoniae | Kpc-Kp1 | 32                            | −     | −     | ++     |
|           | Kpc-Kp6 | 512                           | +++   | −     | −      |
|           | Kp3    | 128                           | +++   | −     | −      |
|           | Kp4    | 8                             | +     | −     | +      |

+, greater killing than monotherapy (indicating the additive benefit of apo-transferrin to a ciprofloxacin- or meropenem-containing combination compared with ciprofloxacin or meropenem monotherapy); −, no measurable effect.

We sought to elucidate the potential for apo-transferrin to ciprofloxacin or meropenem. The addition of apo-transferrin to ciprofloxacin or meropenem suppressed the emergence of resistant mutants when compared with antibiotic monotherapy for all groups tested (Figure 1). The frequency of resistance emergence was greater for A. baumannii than K. pneumoniae strains (Figure 1).

We also selected for resistant mutants by passaging bacteria over 20 days in growth medium containing antibiotics. The antibiotic concentration was increased every 5 days, on the condition that the strain tolerated the increase, since not all passaged bacteria were killed by each antibiotic concentration increase. This scheme allowed for the gradual accumulation of resistance-conferring mutations as strains were continuously exposed to sub-MIC antibiotics, allowing them to evolve and become more resistant. We confirmed that daily passaging in the antibacterial agents increased resistance levels for all A. baumannii and K. pneumoniae strains assayed (Figure 2). Adding apo-transferrin to the antibiotics markedly dampened the emergence of resistance to the antibacterial agents (Figure 2). In most cases, the resulting MIC shift in the presence of transferrin was 10–100-fold lower than in the absence of transferrin and the MICs remained below the susceptibility breakpoints for the drugs in the presence of transferrin. Lastly, we showed that apo-transferrin combination treatment, but not monotherapy, resulted in sterilization of broth cultures (Table 5).

### Discussion

All bacteria must obtain iron from their environment to survive.2–7 Thus, sequestering iron from bacteria is a promising means to inhibit their growth. We sought to elucidate the potential for apo-transferrin-mediated iron sequestration to enhance antimicrobial effects and reduce emergence of resistance to antibiotics commonly used to treat infections caused by Gram-negative bacteria. We found that apo-transferrin had antimicrobial activity against most K. pneumoniae or A. baumannii strains tested and at concentrations that should be achievable in vivo with infusion into patients. Of note, doses of transferrin up to 1040 mg/kg have been infused into transplant patients with no observable toxicities, resulting in marked decreases in unbound iron.24 For a 70 kg adult, such a dose would be anticipated to raise serum transferrin levels by far more than the apo-transferrin MICs we identified (70000 mg/5000 mL of blood volume=14 mg/mL). Addition of apo-transferrin to ciprofloxacin or meropenem demonstrated additive microbial killing during dynamic time–kill assays, although we found no synergy or antagonism between apo-transferrin and the antibiotics for 95% of bacterial strains assayed in static MIC assays.

Adjunct apo-transferrin markedly inhibited the emergence of resistance in K. pneumoniae and A. baumannii exposed to both ciprofloxacin and meropenem. MICs to the antibiotics remained within the susceptible range despite high inoculum plating or serial passage when transferrin was present, but not in the absence of transferrin. In addition to being necessary for oxidative phosphorylation and ATP production, iron also mediates antibiotic-induced, free-radical DNA damage through the Fenton reaction, a mechanism that has previously been shown to promote the formation of
antibiotic-resistant mutants.\textsuperscript{25–27} This mechanism of sequestering iron and limiting the production of harmful reactive oxygen species may therefore be the means by which apo-transferrin inhibits the emergence of antibiotic resistance.

The acquisition of iron is essential for the growth and survival of bacteria, a process that is carried out by multiple—and sometimes redundant—systems. Surprisingly, we found that supplemental free iron had a limited capacity to abrogate the growth inhibition effected by apo-transferrin, despite the fact that each molecule of apo-transferrin binds to only two ions of iron.\textsuperscript{23} While zinc was unable to reverse the effects of apo-transferrin for most strains, it did so for one of the nine tested, underscoring the complexity of metal acquisition and growth inhibition, not only across species but also across strains of the same species. Conversely, addition of haemin was effective at reversing apo-transferrin activity. This finding highlights the important role of haem-binding proteins in an}

\textbf{Figure 1.} Selection of antibiotic-resistant mutants by sub-MIC passage. The addition of apo-transferrin suppressed the emergence of antibiotic-resistant mutants. (a) \textit{K. pneumoniae} or (b) \textit{A. baumannii} was cultured with $\frac{1}{3}$ of the MIC of ciprofloxacin with and without apo-transferrin for 24 h and mutants were selected by plating bacteria on agar plates supplemented with 16 mg/L ciprofloxacin or meropenem. We were unable to select for meropenem-resistant mutants in VA-AB21 or any antibiotic-resistant mutants in AB074 using this method (three attempts). The median (IQR) is plotted for each group.
alternative pathway of iron uptake by A. baumannii and K. pneumoniae: a surreptitious route unaffected by apo-transferrin. Indeed, both organisms are known to express haem uptake systems, capable of extracting iron from host haem even when siderophore and free-iron uptake pathways are stymied.28,29 Thus, to fully realize the therapeutic potential of iron-starvation strategies, it may be necessary to inhibit bacterial haem uptake processes or the activity of the haem oxygenase enzymes that enable bacteria to liberate haem-bound iron.

The variations in transferrin susceptibility and reversal by haem seen across strains is likely due to the well-described variability in strain expression of different iron acquisition mechanisms.4–7,30–32 Individual strains may express different siderophores or haem uptake mechanisms and at different environmental concentrations of iron. Nevertheless, we found variations greater across species than across strains of the same species and, despite the variations, most strains remained susceptible to transferrin at clinically achievable concentrations.

One limitation of the serial passage assays described herein is that the assay evaluates a fixed drug concentration over a short period of time (typically 24 h) and against a small total bacterial burden. In the clinic, drug concentrations change over time, therapy is typically longer than 24 h and the bacterial burden is typically greater. Kinetic models, such as one-compartment or hollow-fibre infection models, which can simulate human drug exposures, therapy duration and clinically relevant bacterial burdens, could be useful to evaluate the propensity for the selection for new resistance or the amplification of pre-existing resistance. Of note, transferrin’s large size as a molecule does limit the ability to conduct hollow-fibre models. Further studies are ongoing, seeking to define the optimal dosage of ciprofloxacin and meropenem with adjunct apo-transferrin therapy.

In conclusion, the addition of apo-transferrin reduces the emergence of antibiotic resistance in key Gram-negative bacterial pathogens. Adjunct apo-transferrin is a promising biological agent, with potential to help preserve the effectiveness of critically needed antibiotics used to treat infections caused by Gram-negative bacteria.

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**Transparency declarations**
None to declare.

**Supplementary data**
Figures S1 to S3 are available as Supplementary data at JAC Online.
Table 5. Stepwise antibiotic increase leads to sterilization of apo-transferrin-containing combinations, but not antibiotic monotherapy treatment groups

| Species          | Strain | Passage (days) | Ciprofloxacin (mg/L) | Ciprofloxacin/ apo-transferrin (mg/L) | Meropenem (mg/L) | Meropenem/ apo-transferrin (mg/L) | Apo-transferrin (mg/L) |
|------------------|--------|----------------|----------------------|---------------------------------------|------------------|----------------------------------|-----------------------|
| *K. pneumoniae*  | KP3    | 1–5            | 0.0078               | 0.0078/256                            | 0.015            | 0.015/256                        | 256                   |
|                  |        | 6–10           | 0.078                | 0.078/512                             | 0.03             | 0.03/512                         | 512                   |
|                  |        | 11–15          | 0.78                 | 0.78/512                              | 0.03             | 0.03/512                         | 512                   |
|                  |        | 16–20          | —                    | —                                     | —                | —                                | —                     |
|                  | KP4    | 1–5            | 0.0078               | 0.0078/8                              | 0.015            | 0.015/8                          | 8                     |
|                  |        | 6–10           | 0.078                | 0.078/16                              | 0.03             | 0.03/16                          | 16                    |
|                  |        | 11–15          | —                    | —                                     | 0.3              | 0.3/16                           | 16                    |
|                  |        | 16–20          | —                    | —                                     | 0.3              | 0.3/32                           | 32                    |
| *A. baumannii*   | AB074  | 1–5            | 0.25                 | 0.25/4                                | 0.125            | 0.125/4                          | 4                     |
|                  |        | 6–10           | 0.5                  | 0.5/8                                 | 0.25             | 0.25/8                           | 8                     |
|                  |        | 11–15          | 5                    | 5/16                                  | 2.5              | 2.5/16                           | 16                    |
|                  |        | 16–20          | —                    | —                                     | 25               | 25/32                            | 32                    |
|                  | VA-AB21| 1–5            | 0.25                 | 0.25/2                                | 0.125            | 0.125/2                          | 2                     |
|                  |        | 6–10           | 0.5                  | 0.5/4                                 | 0.25             | 0.25/4                           | 4                     |
|                  |        | 11–15          | 1                    | 1/8                                   | 2.5              | 2.5/8                            | 8                     |
|                  |        | 16–20          | 50                   | 50/16                                 | 25               | 25/16                            | 16                    |

Ciprofloxacin and meropenem were increased in a dose-dependent manner, with or without the addition of apo-transferrin. The combination of apo-transferrin and antibiotic, but not antibiotic or apo-transferrin alone, resulted in sterilization after passaging. Every fifth passage, the presence of viable bacteria was confirmed by plating bacteria on non-selective agar plates. Non-shaded boxes indicate that growth was observed and shaded boxes indicate the conditions at which no growth was observed.

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