Human recombinant lactoferrin acts synergistically with antimicrobials commonly used in neonatal practice against coagulase-negative staphylococci and *Candida albicans* causing neonatal sepsis

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Neonatal sepsis causes significant mortality and morbidity. Coagulase-negative staphylococci (CoNS) and *Candida* frequently cause neonatal sepsis at >72 h of age. Lactoferrin, which is present in human milk, is a component of innate immunity and has broad-spectrum antimicrobial activity. The synergistic effects of lactoferrin with antibiotics against neonatal isolates have not been systematically evaluated. Here, eight clinical strains (seven neonatal) of CoNS and three strains (two neonatal) of *Candida albicans* were studied. MIC₅₀ and MIC₉₀ values of human recombinant lactoferrin (talactoferrin; TLF), vancomycin (VAN) and nafcillin (NAF) against CoNS, and of TLF, amphotericin B (AMB) and fluconazole (FLC) against *C. albicans*, were evaluated according to established guidelines. Antimicrobial combinations of TLF with NAF or VAN against CoNS, and TLF with AMB or FLC against *C. albicans*, were evaluated by a checkerboard method with serial twofold dilutions. Synergy was evaluated by the median effects principle, and combination indices and dose reduction indices were reported at 50, 75 and 90 % inhibitory effect at several drug-dose ratios. It was found that TLF acted synergistically with NAF and VAN against CoNS, and with AMB and FLC against *C. albicans*, at multiple dose effects and drug-dose ratios with few exceptions. In synergistic combinations, drug reduction indices indicated a significant reduction in doses of antibiotics, which may be clinically relevant. Thus TLF acts synergistically with anti-staphylococcal and anti-*Candida* agents commonly used in neonatal practice and is a promising agent that needs to be evaluated in clinical studies.

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INTRODUCTION

Sepsis is a major cause of death in the neonatal period, especially in preterm and very-low-birth-weight neonates (birth weight <1500 g) (Kaufman & Fairchild, 2004; Lawn *et al.*, 2006). Sepsis significantly increases morbidity, prolongs the need for ventilation and intravascular access, increases the length of hospital stay and increases the incidence of bronchopulmonary dysplasia, necrotizing enterocolitis and adverse neurodevelopmental outcomes (Adams-Chapman & Stoll, 2006; Stoll *et al.*, 2002, 2004). Coagulase-negative staphylococci (CoNS) and *Candida albicans* are among the most common organisms isolated from neonatal sepsis (Stoll *et al.*, 2002). The global emergence of antibiotic resistance (Levy, 1998, 2001) and immature neonatal host defences dictate the need to use

non-antibiotic agents that can enhance host immunity and can be used against neonatal sepsis. Lactoferrin, a component of innate immunity, is one such agent.

Lactoferrin is an 80 kDa, monomeric, diferric, cationic glycoprotein with an isoelectric point of between 8.4 and 9.0 (Moguilevsky *et al.*, 1985; Sanchez *et al.*, 1992) and belongs to the transferrin family of iron-binding glycoproteins. Lactoferrin is composed of 690 aa with a highly conserved three-dimensional structure, and its role in several pathophysiological functions including iron homeostasis, organ morphogenesis, host defence against infection, inflammation and cancer is emerging (Baker *et al.*, 1998; Ward *et al.*, 2005).

Lactoferrin is found on mucosal surfaces, in significant concentrations in human colostrum (7 g l⁻¹) and less so in mature human milk (1 g l⁻¹), tears (3.8 g l⁻¹), saliva (20 mg l⁻¹), seminal fluid and secondary granules of neutrophils (Hennart *et al.*, 1991; Masson *et al.*, 1966, 1969; Masson & Heremans, 1971). Normal serum levels in

Abbreviations: AMB, amphotericin B; CI, combination index; CoNS, coagulase-negative staphylococci; DRI, dose reduction index; FLC, fluconazole; NAF, nafcillin; TLF, human recombinant lactoferrin (talactoferrin alpha); VAN, vancomycin.

humans range from 0.4 to 2 mg l⁻¹ and may increase to 200 mg l⁻¹ following its release from neutrophils during sepsis (Bennett & Kokocinski, 1978). The expression and secretion of lactoferrin in significant concentrations on mucosal surfaces and its release at inflammatory sites by polymorphonuclear neutrophils have established its role as an innate immunity agent, as well as a contributor to acquired cellular and humoral immune responses.

Lactoferrin has broad-spectrum antimicrobial activity against bacteria, fungi, viruses and protozoa. Its antimicrobial activity stems from two distinct effects: (i) its iron-sequestering ability, which can be negated by saturation with iron (Kalmar & Arnold, 1988; Yamauchi *et al.*, 1993); and (ii) its iron-independent killing due to a direct interaction with the microbial surface resulting in cell lysis (Farnaud & Evans, 2003; Orsi, 2004; Valenti & Antonini, 2005).

Bovine lactoferrin has been shown to inhibit planktonic (free-living) organisms, as well as biofilms of *Staphylococcus epidermidis* growing on soft contact lenses *in vitro* (Leitch & Willcox, 1999b). Both bovine and human lactoferrins inhibit *Candida* isolates from the oral cavity of immunocompromised patients by their action on *Candida* cell membranes (Xu *et al.*, 1999) and exhibit synergy with common antifungal agents (Kuipers *et al.*, 1999). Synthetic lactoferrin-derived peptides have been shown to have a greater antifungal effect compared with native lactoferrin *in vitro*, and the first two arginines at the N-terminus of human lactoferrin appear to be critical in the candidacidal activity (Lupetti *et al.*, 2000).

Synergistic effects of lactoferrin with antimicrobial agents against *S. epidermidis* and *Candida* species have been reported (Leitch & Willcox, 1999b; Lupetti *et al.*, 2003), but the term synergy in drug combinations has been loosely applied without definite modelling or derivations, and has not been evaluated at multiple dose effects or drug-dose ratios (Odds, 2003). The utility of combining human recombinant lactoferrin (talactoferrin alpha; TLF) with the commonly used antimicrobials in neonatal practice to enhance antimicrobicidal effects has not yet been reported.

METHODS

Antimicrobials. TLF expressed in *Aspergillus awamori* (Ward *et al.*, 1995) was provided by Agennix. TLF stock solution (100 mg ml⁻¹) was diluted to give serial twofold dilutions starting from a concentration of 8300 µg ml⁻¹. Fluconazole (FLC) stock solution (2 mg ml⁻¹; Sigma-Aldrich) was diluted to give serial twofold dilutions starting from 64 µg ml⁻¹. Amphotericin B (AMB) obtained from *Streptomyces* (Sigma-Aldrich), vancomycin (VAN; USB) and nafcillin (NAF; Sigma-Aldrich) were used in serial twofold dilutions starting from 4 µg ml⁻¹.

Organisms. The organisms used were *S. epidermidis* ATCC 55133, seven clinical strains of CoNS (*S. epidermidis* 100H and 101S, CoNS 102R, 103A, 104O, 105D and 106F) and *C. albicans* strains ATCC 32354, 200C and 201M. All clinical strains (except for *S. epidermidis* ATCC 55133 and *C. albicans* ATCC 32354, which are human

non-neonatal isolates) were isolated from the peripheral blood of neonates with sepsis at Texas Children's Hospital, Houston, TX, USA.

Growth media. Sabouraud dextrose agar plates and glucose/yeast extract/peptone broth (GYEP) were used for *Candida* subcultures, and RPMI 1640 without bicarbonate buffered with 0.165 M MOPS to a pH of 7 was used for antimicrobial susceptibility testing. For CoNS, trypticase soy agar with 5% sheep blood and trypticase soy broth (TSB) were used for subcultures and antimicrobial susceptibility testing, respectively.

Inoculum preparation. CoNS and *C. albicans* were plated overnight from cryogenic stocks stored at -80 °C. Five colonies were picked and inoculated into TSB or GYEP broth, respectively (75 ml in a 250 ml flask), and incubated for up to 2 h in an incubator shaker at 37 °C, resulting in growth-phase organisms. The suspension was centrifuged at 1932 g for 10 min three times and the sediment was suspended in normal saline to an OD₆₃₀ of 0.6 (corresponding to 0.5 × 10⁸–1 × 10⁸ c.f.u. ml⁻¹) for CoNS and an OD₄₉₀ of 0.6 (corresponding to 1 × 10⁷–5 × 10⁷ c.f.u. ml⁻¹) for *C. albicans*. Quantitative cultures of the final suspension confirmed the final concentrations.

Antimicrobial susceptibility testing of CoNS and *C. albicans*

MICs of TLF, NAF and VAN against CoNS. MIC₅₀ and MIC₉₀ values were determined using the broth microdilution method as recommended by the CLSI (2003, 2007) with minor modifications. Five microlitres of a 1:10-diluted inoculum was added to 100 µl of the serial dilutions of the antimicrobial to be tested in a 96-well microtitre plate. The final concentration of CoNS in each well of the microtitre plate was 2.5 × 10⁵–5 × 10⁵ c.f.u. ml⁻¹. Microtitre plates were incubated for 24 h at 35 °C. Drug-free TSB with organisms (growth control) and organism-free drug controls (sterility control) were used for comparison. Microtitre plates were incubated at 35 °C for 24 h, and MIC₅₀ and MIC₉₀ values were estimated by a colorimetric method using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide (XTT). Menadione [10 mM solution in acetone (99.9% HPLC-grade)] was added to XTT (0.5 g l⁻¹) to give a 1 µM concentration of menadione and 100 µl of this was added to the wells of 96-well microtitre plates and incubated in the dark for 2 h. The plates were centrifuged for 5 min at 1932 g, 100 µl supernatant was transferred to a new microtitre plate and the colour was read at A₄₉₀ in a microtitre plate reader (Cerca *et al.*, 2005). End points at 50 and 90% inhibition of growth compared with the growth control were determined as MIC₅₀ and MIC₉₀ values, respectively. Experiments were carried out in duplicate on two different days and the means of the readings of XTT reduction were used to determine the MIC₅₀ and MIC₉₀.

MICs of TLF, AMB and FLC against *C. albicans*. MIC₅₀ and MIC₉₀ values were determined using the broth microdilution method as described by the CLSI (2002). *C. albicans* inoculum (100 µl) was added to 100 µl of serial twofold dilutions of the antifungal agent to be tested in 96-well microtitre plates. The final concentration of *C. albicans* was 0.25 × 10³–0.5 × 10³ c.f.u. ml⁻¹ in each well of the microtitre plate. Drug-free medium with organisms (growth control) and organism-free drug controls (sterility control) were used for comparison. Microtitre plates were incubated at 35 °C for 46 h, and MIC₅₀ and MIC₉₀ values were estimated by a colorimetric method using XTT as described above. Experiments were carried out in duplicate on two different days and the means of the readings of XTT reduction were used to determine MIC₅₀ and MIC₉₀ values.

Antimicrobial susceptibility testing of CoNS and *C. albicans* using antimicrobial combinations with TLF. Antimicrobial combinations of TLF with VAN or NAF were tested against CoNS, and combinations

of TLF with AMB or FLC against *C. albicans*. An 8 × 8 chequerboard matrix of the drugs (50 µl each) in serial twofold dilutions including zero concentrations on the x- and the y-axes were set up in 96-well microtitre plates. As in the MIC determinations, 5 µl CoNS suspension (2.5×10^4 – 7.5×10^4 c.f.u. ml⁻¹) or 100 µl *C. albicans* suspension (0.5×10^3 – 1×10^3 c.f.u. ml⁻¹) was added to the wells containing the antimicrobial combinations. The plates were incubated at 35 °C for 24 h for CoNS and for 46 h for *C. albicans*. Inhibitory end points were determined by XTT reduction. Experiments were performed in duplicate on two different days and the means of readings of XTT reduction on each day were used to provide two data points for analyses.

Analyses of drug interactions in antimicrobial combinations. The inhibitory effects of the antimicrobials in combination with TLF were tabulated in constant ratios of each other including ratios of their MIC₅₀ values (equipotency ratios). The median effects principle was used to study drug interactions and estimate combination indices (CIs) and dose reduction indices (DRIs) (Chou, 2006).

The median effect equation is given by $f_a/f_u = (D/D_m)^m$, where f_a is the fraction affected by the drug, f_u is the fraction unaffected ($f_u = 1 - f_a$), D is the concentration of the drug, D_m is the median-effect dose (the dose of the drug that produces 50 % of the effect, ED₅₀) and m is the co-efficient signifying the shape of the dose–effect relationship, where $m=1$, $m > 1$ and $m < 1$ indicate hyperbolic, sigmoidal and flat-sigmoidal dose–effect curves, respectively.

The CI value is given by the equation $CI = D_1/D_{x1} + D_2/D_{x2}$, and the DRI for drug 1 = D_{x1}/D_1 and for drug 2 = D_{x2}/D_2 , where D_1 and D_2 are doses of drug 1 and drug 2 in combination and D_{x1} and D_{x2} are doses of drug 1 and drug 2 that produce $x\%$ effect when used alone. A value of $CI < 1$ indicates synergy, $CI > 1$ indicates antagonism and $CI = 1$ indicates an additive effect. The DRI determines by how many fold a drug dose can be reduced in a synergistic drug combination for a given inhibitory effect on the organism. Multiple drug-dose effect calculations were performed using CalcuSyn software (Biosoft) using the constant ratios of the drug combinations.

RESULTS AND DISCUSSION

Antimicrobial susceptibility of clinical strains of CoNS and *C. albicans*

The MIC₅₀ values of TLF against CoNS ranged from 500 to 2000 µg ml⁻¹, but the MIC₉₀ values were beyond the range tested for all strains except CoNS 106F (Table 1). For *C. albicans*, the MIC₅₀ values of TLF ranged from 62.5 to 250 µg ml⁻¹, but the MIC₉₀ values were beyond the range tested for all three strains. The huge difference in concentrations between MIC₅₀ and MIC₉₀ values suggest a bacteriostatic effect rather than a bacteriocidal effect. Similar large differences were seen with the fungistatic FLC, where the MIC₉₀ values were 1000 times greater than the MIC₅₀ values. The differences between MIC₅₀ and MIC₉₀ for NAF (varying from the same concentration to 16-fold), vancomycin (same to 2-fold) and amphotericin (16–33-fold) were not as pronounced as that of TLF or FLC.

Cationic lactoferrin probably acts by binding to anionic lipotechoic acid of *S. epidermidis*, thereby reducing its charge and allowing lysozyme to act (Leitch & Willcox, 1999a). Lactoferrin at 1800 µg ml⁻¹ together with lysozyme has been shown to be effective *in vitro* against *S. epidermidis*. In addition, lactoferrin alone at 200 µg ml⁻¹ and in conjunction at 100 µg ml⁻¹ with azole group antifungal agents inhibits *C. albicans* (Wakabayashi *et al.*, 1996, 1998). In our experiments, we found that TLF alone inhibited (MIC₅₀) all strains of CoNS at concentrations of 500–2000 µg ml⁻¹ and all strains of *C. albicans* at concentrations of 62.5–250 µg ml⁻¹, which is similar to previously published studies (Lupetti *et al.*, 2000, 2003).

Table 1. Antimicrobial susceptibility of clinical strains of CoNS and *C. albicans*

Antimicrobial susceptibility performed using the microdilution method in 96-well microtitre plates and an XTT reduction assay was used to determine inhibitory end points.

Clinical strain	TLF (µg ml ⁻¹)		VAN (µg ml ⁻¹)		NAF (µg ml ⁻¹)	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
<i>S. epidermidis</i> ATCC 55133	500	>8300	0.125	0.25	0.125	2
<i>S. epidermidis</i> 100H	500	>8300	1	2	0.125	1
<i>S. epidermidis</i> 101S	500	>8300	2	4	0.0625	0.0625
CoNS 102R	500	>8300	1	2	0.032	0.125
CoNS 103A	2000	>8300	1	2	0.032	0.0625
CoNS 104O	500	>8300	1	2	0.032	0.0625
CoNS 105D	500	>8300	1	2	0.0625	1
CoNS 106F	1000	8300	2	2	0.032	0.032
	FLC (µg ml ⁻¹)		AMB (µg ml ⁻¹)			
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
<i>C. albicans</i> ATCC 32354	62.5	>8300	0.032	32	0.03	1
<i>C. albicans</i> 200C	250	>8300	0.25	32	0.0625	1
<i>C. albicans</i> 201M	250	>8300	0.25	32	0.0625	1

Lactoferrin may act in concert with other human defence mechanisms *in vivo* and may have an enhanced antimicrobial effect (Valenti & Antonini, 2005). In a neonatal rat model of polymicrobial infection of *S. epidermidis* and *C. albicans*, TLF significantly improved survival (Venkatesh *et al.*, 2007). Other investigators have reported the efficacy of TLF in animal models of systemic *Escherichia coli* infection (Edde *et al.*, 2001) and of synthetic lactoferrin-derived peptides in invasive *Candida* infections (Lupetti *et al.*, 2007).

Antimicrobial combinations with TLF

There are many advantages of combining drugs in the treatment of infections, including enhancing efficacy, the ability to decrease the doses of antimicrobial agents (leading to a reduction in toxicity) and minimizing the development of resistance. The potential for the clinical use of lactoferrin in conjunction with antibiotics in the therapy or prevention of neonatal sepsis suggested that it would be useful to evaluate the drug interactions of TLF with antibiotics commonly used in neonatal practice.

Although many methods of evaluating synergy, an additive effect or antagonism in drug combinations exist, we used the median effects principle elucidated by Chou (2006), which is widely used in cancer and infection research (Berenbaum, 1989; Greco *et al.*, 1995; Odds, 2003; Prichard *et al.*, 1993). The advantage of this method is that it overcomes the assumption that drug interactions are linear across dosages and drug effects. There is no general equation that fits all of the dose–response curves and hence the need exists to evaluate results over a range of effects (50–90 % inhibition) (Martinez-Irujo *et al.*, 1996). We evaluated drug combinations with TLF in a systematic manner at three different dose effects (ED₅₀, ED₇₅ and ED₉₀) and multiple drug-dose ratios that may be relevant clinically. We confirmed that our data fitted the median effects equation (mean *r* value ≥ 0.95), rendering calculation of the CIs and DRIs valid (Chou, 2006).

CIs for drug combinations with TLF. In the eight strains of CoNS tested, the TLF and NAF combination was synergistic (CI <1) over a wide range of drug-dose ratios at dose effects of 50, 75 and 90 % with very few exceptions (all exceptions were at ED₅₀: *S. epidermidis* ATCC 55133 at a ratio of 8000:1 and CoNS 103A at 64 000:1 and 32 000:1; Table 2). Similarly, the TLF and VAN combination was synergistic (CI <1) at multiple drug-dose ratios at ED₅₀, ED₇₅ and ED₉₀ with no exceptions.

In the three strains of *C. albicans* tested, the TLF and AMB combination was synergistic (CI <1) over a wide range of drug-dose ratios at ED₅₀, ED₇₅ and ED₉₀ with no exceptions (Table 3). Similarly, the TLF and FLC combination was synergistic (CI <1) at multiple drug-dose ratios at ED₅₀, ED₇₅ and ED₉₀ with only one exception (ED₅₀: *C. albicans* ATCC 32354 at a ratio of 8000:1).

Thus TLF acted synergistically with NAF and VAN against CoNS including *S. epidermidis*, and with AMB and FLC against *C. albicans*, at most drug-dose ratios with very few exceptions. These exceptions may be explained by the inherent variability of the checkerboard method due to variability in the assay conditions on different days.

The synergy of lactoferrin with antibiotics commonly used against isolates of organisms of clinical interest is of great clinical relevance, as it has the potential for more effective therapy against drug-resistant strains and for a reduction in drug dosage. Leitch & Willcox (1999b) reported that 1024 µg lactoferrin ml⁻¹ reduced the minimum bactericidal concentrations of VAN required to kill *S. epidermidis* twofold. Kuipers *et al.* (1999) reported significant co-operative activity of lactoferrin and apolactoferrin (iron-free) with FLC, AMB and 5-fluorocytosine against clinical *Candida* isolates. Peptides derived from human lactoferrin containing the first 11 N-terminal residues were effective in making fluconazole-resistant *C. albicans* sensitive to fluconazole (Leitch & Willcox, 1999a; Wakabayashi *et al.*, 1996, 1998).

The mechanism of the synergistic effect of lactoferrin with antifungal and anti-staphylococcal agents has not been fully validated. It appears that the synergistic effect is not due to the iron-sequestering effects of lactoferrin, as both apolactoferrin and iron-saturated lactoferrin have similar activities *in vitro* (Kuipers *et al.*, 1999). A direct effect on the cell membranes of *Candida* and staphylococci is more likely (Leitch & Willcox, 1999a; Xu *et al.*, 1999). We speculate that the action of lactoferrin on cell membranes complements the action of other drugs and may be responsible for the synergy.

DRIs for drug combinations with TLF. For CoNS, the DRIs indicated a significant reduction in the doses of NAF and VAN required when used in combination with TLF for a given effect, but varied widely among the strains of CoNS tested and also among the different drug-dose ratios tested in a single strain (Table 4). The DRI ranges for NAF in combination with TLF were: 2.5–10.4 against *S. epidermidis* ATCC 55133; 5.3–14.3 against *S. epidermidis* 100H; 2.1–6.6 against *S. epidermidis* 101S; 11.7–176 against CoNS 102R; 1.7–7.3 against CoNS 103A; 1.7–4.3 against CoNS 104O; 3.7–90.3 against CoNS 105D; and 1.5–4.2 against CoNS 106F. The DRI ranges for VAN in combination with TLF were: 1.6–5.9 against *S. epidermidis* ATCC 55133; 2.2–6.4 against *S. epidermidis* 100H; 2.3–4.8 against *S. epidermidis* 101S; 2.7–9.2 against CoNS 102R; 1.2–3.2 against CoNS 103A; 1.6–4.1 against CoNS 104O; 1.8–4.2 against CoNS 105D; and 1.7–4.9 against CoNS 106F.

For *C. albicans*, the DRIs indicated a significant reduction in the doses of AMB and FLC required when used in combination with TLF for a given effect, but varied widely among the strains of *C. albicans* tested and also among the different drug-dose ratios tested in a single strain (Table 5). The DRI ranges for AMB in combination with TLF were:

Table 2. CIs for drug combinations with TLF against CoNS

CIs were derived using the median effects principle. The effect was synergistic for CI <1, additive for CI=1 and antagonistic for CI >1. Results are shown as means (SD). TLF was synergistic across most drug-dose ratios (exceptions indicated in bold) and for different dose effects against clinical isolates of CoNS. ND, Not done.

	TLF:NAF ratio	CI for TLF with NAF			TLF:VAN ratio	CI for TLF with VAN		
		ED ₅₀	ED ₇₅	ED ₉₀		ED ₅₀	ED ₇₅	ED ₉₀
<i>S. epidermidis</i> ATCC 55133	32 000:1	0.29 (0.05)	0.19 (0.05)	0.13 (0.04)	4000:1*	0.48 (0.14)	0.39 (0.12)	0.42 (0.18)
	16 000:1	0.25 (0.04)	0.18 (0.04)	0.14 (0.03)	2000:1	0.54 (0.25)	0.36 (0.02)	0.31 (0.08)
	8000:1	1.04 (1.16)	0.44 (0.36)	0.21 (0.07)	1000:1	0.49 (0.13)	0.40 (0.01)	0.38 (0.09)
	4000:1*	0.23 (0.02)	0.23 (0.02)	0.25 (0.06)	500:1	0.50 (0.13)	0.47 (0.01)	0.49 (0.08)
	2000:1	0.73 (0.65)	0.44 (0.28)	0.28 (0.08)	250:1	0.48 (0.16)	0.33 (0.13)	0.24 (0.12)
	1000:1	0.33 (0.19)	0.37 (0.17)	0.42 (0.13)	125:1	0.65 (0.15)	0.55 (0.03)	0.50 (0.16)
<i>S. epidermidis</i> 100H	8000:1	0.41 (0.01)	0.22 (0.07)	0.17 (0.05)	2000:1	0.39 (0.00)	0.33 (0.03)	0.38 (0.05)
	4000:1*	0.43 (0.01)	0.24 (0.06)	0.15 (0.07)	1000:1	0.41 (0.08)	0.35 (0.10)	0.37 (0.06)
	2000:1	0.40 (0.09)	0.25 (0.10)	0.18 (0.08)	500:1*	0.44 (0.15)	0.40 (0.16)	0.40 (0.13)
	1000:1	0.39 (0.15)	0.27 (0.10)	0.21 (0.05)	250:1	0.48 (0.18)	0.44 (0.13)	0.43 (0.07)
	500:1	0.29 (0.05)	0.25 (0.12)	0.24 (0.15)	125:1	0.53 (0.20)	0.48 (0.12)	0.45 (0.03)
<i>S. epidermidis</i> 101S	16 000:1	0.46 (0.31)	0.25 (0.09)	0.16 (0.02)	2000:1	0.44 (0.25)	0.30 (0.13)	0.25 (0.09)
	8000:1*	0.43 (0.24)	0.29 (0.09)	0.22 (0.03)	1000:1	0.36 (0.11)	0.29 (0.08)	0.27 (0.09)
	4000:1	0.32 (0.01)	0.29 (0.0)	0.27 (0.0)	500:1	0.39 (0.10)	0.32 (0.09)	0.28 (0.10)
	2000:1	0.41 (0.02)	0.35 (0.01)	0.31 (0.01)	250:1*	0.43 (0.09)	0.36 (0.08)	0.32 (0.08)
	1000:1	0.50 (0.05)	0.42 (0.02)	0.36 (0.0)	125:1	0.48 (0.13)	0.41 (0.09)	0.36 (0.08)
CoNS 102R	16 000:1*	0.13 (0.16)	0.17 (0.02)	0.66 (0.71)	2000:1	0.44 (0.15)	0.38 (0.04)	0.36 (0.18)
	8000:1	0.12 (0.10)	0.15 (0.02)	0.26 (0.17)	1000:1	0.38 (0.11)	0.32 (0.00)	0.28 (0.06)
	4000:1	0.11 (0.09)	0.11 (0.04)	0.13 (0.01)	500:1*	0.38 (0.08)	0.32 (0.02)	0.28 (0.02)
	2000:1	0.12 (0.09)	0.12 (0.04)	0.14 (0.00)	250:1	0.41 (0.02)	0.34 (0.01)	0.30 (0.01)
	1000:1	0.13 (0.09)	0.12 (0.04)	0.13 (0.01)	125:1	0.44 (0.07)	0.40 (0.03)	0.37 (0.01)
CoNS 103A	64 000:1*	1.24 (0.32)	0.33 (0.03)	0.17 (0.03)	2000:1*	0.63 (0.26)	0.37 (0.03)	0.32 (0.01)
	32 000:1	1.05 (0.32)	0.33 (0.02)	0.18 (0.04)	1000:1	0.93 (0.68)	0.47 (0.19)	0.33 (0.07)
	16 000:1	0.83 (0.16)	0.35 (0.00)	0.23 (0.03)	500:1	0.55 (0.08)	0.42 (0.0)	0.37 (0.01)
	8000:1	0.64 (0.06)	0.37 (0.00)	0.28 (0.02)	250:1	0.58 (0.03)	0.45 (0.02)	0.39 (0.02)
	4000:1	0.51 (0.02)	0.39 (0.02)	0.36 (0.01)	125:1	0.61 (0.08)	0.48 (0.02)	0.40 (0.0)
CoNS 104O	2000:1	0.55 (0.00)	0.45 (0.01)	0.40 (0.03)	62.5:1	0.86 (0.06)	0.62 (0.06)	0.45 (0.04)
	1000:1	0.63 (0.03)	0.52 (0.01)	0.45 (0.01)	ND	ND	ND	ND
	16 000:1*	0.57 (0.04)	0.33 (0.02)	0.25 (0.06)	2000:1	0.39 (0.02)	0.42 (0.20)	0.58 (0.50)
	8000:1	0.52 (0.02)	0.36 (0.02)	0.30 (0.09)	1000:1	0.40 (0.06)	0.49 (0.23)	0.68 (0.50)
	4000:1	0.52 (0.05)	0.41 (0.03)	0.37 (0.13)	500:1*	0.43 (0.04)	0.53 (0.20)	0.70 (0.44)
CoNS 105D	2000:1	0.53 (0.04)	0.47 (0.02)	0.44 (0.09)	250:1	0.50 (0.03)	0.55 (0.19)	0.65 (0.39)
	1000:1	0.62 (0.04)	0.56 (0.03)	0.52 (0.09)	125:1	0.56 (0.01)	0.64 (0.20)	0.77 (0.44)
	16 000:1	0.55 (0.17)	0.24 (0.1)	0.12 (0.08)	2000:1	0.47 (0.12)	0.35 (0.10)	0.37 (0.24)
	8000:1*	0.45 (0.20)	0.22 (0.10)	0.12 (0.07)	1000:1	0.44 (0.06)	0.35 (0.14)	0.36 (0.26)
	4000:1	0.40 (0.23)	0.21 (0.11)	0.12 (0.07)	500:1*	0.44 (0.02)	0.40 (0.14)	0.43 (0.28)
CoNS 106F	2000:1	0.40 (0.18)	0.25 (0.10)	0.17 (0.06)	250:1	0.49 (0.05)	0.45 (0.15)	0.45 (0.23)
	1000:1	0.45 (0.21)	0.31 (0.12)	0.22 (0.08)	125:1	0.59 (0.03)	0.53 (0.18)	0.50 (0.30)
	32 000:1*	0.98 (0.48)	0.37 (0.07)	0.25 (0.01)	2000:1	0.42 (0.03)	0.31 (0.02)	0.26 (0.06)
	16 000:1	0.74 (0.18)	0.35 (0.05)	0.25 (0.05)	1000:1	0.44 (0.01)	0.33 (0.03)	0.28 (0.04)
	8000:1	0.66 (0.07)	0.40 (0.02)	0.31 (0.04)	500:1*	0.45 (0.0)	0.36 (0.02)	0.31 (0.04)
	4000:1	0.45 (0.02)	0.32 (0.14)	0.26 (0.09)	250:1	0.49 (0.01)	0.41 (0.04)	0.35 (0.07)
	2000:1	0.66 (0.04)	0.47 (0.01)	0.36 (0.01)	125:1	0.61 (0.05)	0.49 (0.02)	0.40 (0.07)
	1000:1	0.71 (0.03)	0.51 (0.05)	0.39 (0.06)	62.5:1	0.69 (0.32)	0.46 (0.22)	0.30 (0.15)

*Represents equipotency ratios (ratios of MIC₅₀).

Table 3. CIs for drug combinations with TLF against *C. albicans*

CIs were derived by the median effects principle. The effect was synergistic for CI <1, additive for CI=1 and antagonistic for CI >1. Results are shown as means (SD). TLF was synergistic across most drug-dose ratios (exception indicated in bold) and for different dose effects against clinical isolates of *C. albicans*. ND, Not done.

Organism	TLF:AMB ratio	CI for TLF with AMB			TLF:FLC ratio	CI for TLF with FLC		
		ED ₅₀	ED ₇₅	ED ₉₀		ED ₅₀	ED ₇₅	ED ₉₀
<i>C. albicans</i> ATCC 32354	8000:1	0.07 (0.05)	0.07 (0.06)	0.07 (0.05)	8000:1	1.04 (0.89)	0.24 (0.02)	0.08 (0.06)
	4000:1	0.08 (0.08)	0.07 (0.07)	0.08 (0.05)	4000:1	0.79 (0.64)	0.21 (0.01)	0.09 (0.08)
	2000:1*	0.11 (0.08)	0.11 (0.08)	0.12 (0.05)	2000:1*	0.47 (0.21)	0.19 (0.09)	0.11 (0.12)
	1000:1	0.11 (0.08)	0.12 (0.08)	0.14 (0.07)	1000:1	0.38 (0.05)	0.18 (0.11)	0.11 (0.12)
	500:1	0.09 (0.11)	0.10 (0.11)	0.12 (0.10)	500:1	0.37 (0.06)	0.20 (0.14)	0.13 (0.14)
	250:1	0.21 (0.29)	0.16 (0.23)	0.13 (0.17)	250:1	0.32 (0.07)	0.22 (0.15)	0.17 (0.17)
	125:1	0.27 (0.38)	0.20 (0.28)	0.15 (0.21)	ND	ND	ND	ND
	62.5:1	0.57 (0.80)	0.33 (0.46)	0.20 (0.25)	ND	ND	ND	ND
<i>C. albicans</i> 200C	8000:1	0.22 (0.09)	0.14 (0.03)	0.09 (0.01)	8000:1	0.13 (0.03)	0.06 (0.03)	0.03 (0.03)
	4000:1*	0.23 (0.12)	0.15 (0.03)	0.11 (0.03)	4000:1	0.11 (0.03)	0.06 (0.03)	0.03 (0.03)
	2000:1	0.30 (0.18)	0.18 (0.03)	0.13 (0.04)	2000:1	0.11 (0.03)	0.05 (0.03)	0.03 (0.03)
	1000:1	0.20 (0.07)	0.16 (0.07)	0.14 (0.08)	1000:1*	0.12 (0.03)	0.06 (0.05)	0.04 (0.04)
	500:1	0.13 (0.12)	0.15 (0.12)	0.17 (0.13)	500:1	0.13 (0.07)	0.07 (0.05)	0.04 (0.04)
	250:1	0.21 (0.11)	0.21 (0.13)	0.21 (0.15)	250:1	0.15 (0.10)	0.08 (0.07)	0.04 (0.04)
<i>C. albicans</i> 201M	8000:1	0.41 (0.02)	0.24 (0.01)	0.18 (0.00)	8000:1	0.35 (0.21)	0.06 (0.01)	0.02 (0.01)
	4000:1*	0.38 (0.06)	0.26 (0.01)	0.21 (0.02)	4000:1	0.18 (0.04)	0.05 (0.02)	0.02 (0.01)
	2000:1	0.40 (0.04)	0.30 (0.01)	0.23 (0.00)	2000:1	0.15 (0.03)	0.05 (0.03)	0.02 (0.01)
	1000:1	0.46 (0.05)	0.35 (0.02)	0.27 (0.01)	1000:1*	0.15 (0.07)	0.06 (0.04)	0.03 (0.02)
	500:1	0.73 (0.17)	0.49 (0.09)	0.34 (0.05)	500:1	0.23 (0.13)	0.09 (0.06)	0.04 (0.03)
	250:1	0.35 (0.21)	0.31 (0.15)	0.29 (0.10)	250:1	0.32 (0.24)	0.13 (0.10)	0.05 (0.04)

*Represents equipotency ratios (ratios of MIC₅₀).

8.2–2960 against *C. albicans* ATCC 32354; 4.3–12.7 for *C. albicans* 200C; and 1.5–5.6 for *C. albicans* 201M. The DRIs for FLC in combination with TLF were: 4.1–820 against *C. albicans* ATCC 32354; 8.8–177.5 against *C. albicans* 200C; and 4.9–77.5 against *C. albicans* 201M.

We chose to report DRIs, which estimate the degree by which antibiotic dosage can be reduced in a synergistic drug combination at a given inhibitory effect. The DRIs were significant but variable among the different strains of organism tested and within the same strain at different drug-dose ratios and drug effects. The DRIs indicated twofold to several hundred-fold reductions in drug doses required and may indicate an advantage of using TLF in conjunction with antibiotics in the treatment or prevention of neonatal sepsis. DRIs are not commonly reported in the literature, but the clinical relevance underlines their importance.

Limitations of the study

The limitations of this study follow the inherent variability of checkerboard assays and hence the need for repeated assays. We repeated our experiments in duplicate and on two different days, and the readings on any particular day

were carried out under the same experimental conditions and were averaged, generating two sets of data for analysis. Checkerboard combinations used serial twofold dilutions of the drug, but the effects of the drug concentrations between the twofold dilutions were not evaluated, which may be valuable in future studies. We did not test concentrations higher than 8300 µg ml⁻¹ for TLF, although some studies using higher concentrations (Kuipers *et al.*, 1999) have shown complete inhibition of *Candida* isolates. The general limitation of experiments *in vitro* in terms of correlation with effects *in vivo* that exist may be due to the physical environment as well as the participation of other host defence mechanisms. Antimicrobial combinations need to be evaluated in appropriate animal models using clinical isolates from organisms of interest.

Conclusions

The antimicrobial activity of lactoferrins in general, and TLF in particular, against common neonatal isolates of CoNS (including *S. epidermidis*) and *C. albicans*, and the synergistic effect with antibiotics commonly used in neonatal practice, make TLF a promising agent in the

Table 4. DRIs for drug combinations with TLF against CoNS

DRIs were determined using the median effects principle. Results are shown as means (SD). The DRIs suggest that a significant reduction in antibiotic dosage is possible when combined with TLF across drug-dose ratios and for different dose effects against CoNS. ND, Not done.

Organism	TLF: NAF ratio	DRI for NAF with TLF			TLF: VAN ratio	DRI for VAN with TLF		
		ED ₅₀	ED ₇₅	ED ₉₀		ED ₅₀	ED ₇₅	ED ₉₀
<i>S. epidermidis</i> ATCC 55133	32 000:1	7.10 (5.23)	8.57 (5.19)	10.44 (4.87)	4000:1*	5.92 (0.54)	4.27 (0.67)	3.18 (1.25)
	16 000:1	6.09 (3.24)	7.07 (2.92)	8.23 (2.44)	2000:1	2.43 (2.59)	2.13 (2.02)	1.88 (1.53)
	8000:1	3.53 (4.14)	4.09 (3.66)	5.40 (2.13)	1000:1	2.99 (0.28)	2.88 (0.17)	2.89 (0.72)
	4000:1*	4.92 (0.17)	4.56 (0.79)	4.35 (1.20)	500:1	2.50 (0.42)	2.30 (0.00)	2.14 (0.37)
	2000:1	2.47 (2.36)	2.95 (1.92)	3.74 (1.05)	250:1	2.46 (0.66)	3.39 (1.25)	4.66 (2.21)
	1000:1	3.80 (2.27)	3.05 (1.49)	2.51 (0.86)	125:1	1.64 (0.34)	1.83 (0.10)	2.13 (0.67)
<i>S. epidermidis</i> 100H	8000:1	14.29 (9.37)	10.78 (4.54)	8.41 (1.34)	2000:1	6.36 (1.61)	4.44 (0.09)	3.22 (0.94)
	4000:1*	11.06 (9.02)	11.08 (7.97)	11.17 (6.85)	1000:1	4.50 (1.70)	3.63 (0.80)	2.98 (0.18)
	2000:1	7.91 (6.52)	7.77 (5.37)	7.73 (4.17)	500:1*	3.37 (1.46)	3.03 (1.09)	2.70 (0.71)
	1000:1	5.93 (4.78)	5.66 (3.30)	5.59 (1.79)	250:1	2.71 (1.17)	2.53 (0.72)	2.40 (0.32)
	500:1	5.31 (2.64)	5.35 (3.07)	5.42 (3.50)	125:1	2.23 (0.91)	2.22 (0.52)	2.25 (0.12)
	16 000:1	3.95 (0.91)	5.10 (0.56)	6.64 (0.06)	2000:1	4.49 (0.16)	4.63 (0.66)	4.78 (1.15)
<i>S. epidermidis</i> 101S	8000:1*	3.33 (0.94)	3.93 (0.66)	4.76 (0.34)	1000:1	4.00 (0.29)	4.07 (0.46)	4.17 (1.18)
	4000:1	3.58 (0.54)	3.71 (0.29)	3.83 (0.04)	500:1	3.25 (0.21)	3.53 (0.67)	3.95 (1.34)
	2000:1	2.68 (0.11)	2.92 (0.17)	3.23 (0.18)	250:1*	2.62 (0.25)	2.94 (0.51)	3.32 (0.83)
	1000:1	2.10 (0.14)	2.43 (0.10)	2.79 (0.10)	125:1	2.27 (0.46)	2.55 (0.5)	2.88 (0.59)
	16 000:1*	176 (219.84)	44.78 (39.90)	14.50 (2.12)	2000:1	9.17 (5.00)	8.80 (6.23)	8.61 (7.34)
	8000:1	48.01 (45.24)	29.35 (23.27)	18.19 (11.04)	1000:1	6.66 (2.74)	6.31 (2.67)	6.56 (3.45)
CoNS 102R	4000:1	32.71 (28.98)	28.39 (24.48)	24.62 (20.62)	500:1*	4.80 (1.41)	4.85 (1.35)	4.89 (1.28)
	2000:1	17.94 (13.66)	17.00 (12.31)	16.11 (11.02)	250:1	3.51 (1.27)	3.71 (0.83)	4.01 (0.40)
	1000:1	13.29 (9.78)	12.31 (7.06)	11.65 (4.60)	125:1	2.73 (0.10)	2.84 (0.09)	2.99 (0.15)
	64 000:1*	4.61 (0.66)	5.79 (1.22)	7.27 (2.01)	2000:1*	3.24 (0.65)	3.23 (0.47)	3.21 (0.28)
	32 000:1	3.23 (0.10)	4.41 (0.67)	6.07 (1.63)	1000:1	1.97 (0.77)	2.49 (0.74)	3.16 (0.64)
	16 000:1	2.63 (0.19)	3.49 (0.40)	4.63 (0.71)	500:1	2.31 (0.22)	2.51 (0.18)	2.73 (0.12)
CoNS 103A	8000:1	2.51 (0.20)	3.03 (0.24)	3.66 (0.28)	250:1	2.38 (0.39)	2.27 (0.16)	2.61 (0.15)
	4000:1	2.58 (0.39)	2.69 (0.24)	2.82 (0.09)	125:1	1.77 (0.11)	2.11 (0.06)	2.51 (0.01)
	2000:1	2.09 (0.12)	2.28 (0.00)	2.50 (0.15)	62.5:1	1.20 (0.12)	1.63 (0.16)	2.21 (0.21)
	1000:1	1.70 (0.04)	1.95 (0.00)	2.24 (0.03)	ND	ND	ND	ND
	16 000:1*	3.35 (1.63)	3.67 (0.66)	4.25 (0.63)	2000:1	4.13 (0.61)	3.41 (1.99)	3.09 (2.82)
	8000:1	2.83 (1.18)	3.14 (0.19)	3.56 (0.90)	1000:1	3.35 (0.91)	2.60 (1.41)	2.13 (1.59)
CoNS 104O	4000:1	2.42 (0.73)	2.58 (0.02)	2.97 (1.03)	500:1*	2.69 (0.40)	2.15 (0.92)	1.83 (1.17)
	2000:1	2.10 (0.42)	2.20 (0.00)	2.31 (0.40)	250:1	2.16 (0.23)	2.01 (0.73)	1.87 (1.23)
	1000:1	1.72 (0.180)	1.84 (0.09)	1.96 (0.35)	125:1	1.85 (0.07)	1.67 (0.53)	1.55 (0.91)
	16 000:1	15.51 (14.84)	36.57 (43.03)	90.27 (117)	2000:1	4.21 (0.59)	3.86 (1.64)	3.67 (2.50)
	8000:1*	11.34 (10.84)	23.50 (26.16)	48.21 (58.53)	1000:1	3.33 (0.47)	3.60 (1.69)	4.08 (3.08)
	4000:1	8.86 (8.54)	15.87 (16.45)	28.37 (31.15)	500:1*	2.84 (0.34)	2.83 (1.18)	2.98 (1.96)
CoNS 105D	2000:1	5.43 (4.21)	8.04 (6.45)	11.98 (9.93)	250:1	2.31 (0.44)	2.42 (0.88)	2.56 (1.36)
	1000:1	3.65 (2.47)	5.09 (3.41)	6.99 (4.54)	125:1	1.81 (0.16)	2.04 (0.76)	2.43 (1.45)
	32 000:1*	3.07 (0.10)	3.58 (0.26)	4.21 (0.43)	2000:1	4.47 (1.94)	4.69 (2.10)	4.91 (2.27)
	16 000:1	2.69 (0.41)	3.30 (0.01)	4.17 (0.75)	1000:1	2.77 (1.80)	3.05 (1.35)	3.43 (0.75)
	8000:1	2.25 (0.35)	2.74 (0.06)	3.35 (0.36)	500:1*	2.17 (1.37)	2.39 (1.12)	2.67 (0.80)
	4000:1	3.16 (1.78)	3.62 (1.72)	4.19 (1.55)	250:1	1.70 (1.13)	1.93 (0.89)	2.25 (0.50)
CoNS 106F	2000:1	1.72 (0.03)	2.19 (0.01)	2.77 (0.04)	125:1	1.76 (0.23)	2.10 (0.01)	2.56 (0.34)
	1000:1	1.50 (0.00)	1.99 (0.16)	2.62 (0.40)	62.5:1	1.67 (0.81)	2.51 (1.28)	3.80 (1.97)

*Represents equipotency ratios (ratios of MIC₅₀).

Table 5. DRIs for drug combinations with TLF against *C. albicans*

DRIs were determined using the median effects principle. Results are shown as means (SD). The DRIs suggest that a significant reduction in antibiotic dosages is possible when combined with TLF across various drug-dose ratios and for different dose effects against clinical isolates of *C. albicans*.

Organism	TLF:AMB ratio	DRI for AMB with TLF			TLF:FLC ratio	DRI for FLC with TLF		
		ED ₅₀	ED ₇₅	ED ₉₀		ED ₅₀	ED ₇₅	ED ₉₀
<i>C. albicans</i> ATCC 32354	8000:1	83.6 (103.80)	38.53 (40.26)	19.21 (13.14)	8000:1	7.2 (2.54)	21.39 (19.00)	72.91 (86.40)
	4000:1	82.5 (105.20)	36.63 (39.70)	17.82 (12.42)	4000:1	70.41 (92.8)	213.80 (293)	819.5 (1146)
	2000:1*	26.15 (28.20)	14.65 (11.82)	8.94 (3.62)	2000:1*	5.70 (2.26)	14.34 (13.40)	42.03 (51.20)
	1000:1	16.97 (15.30)	11.64 (8.42)	8.18 (4.13)	1000:1	4.96 (2.32)	11.54 (10.30)	29.55 (34.00)
	500:1	58.90 (75.10)	26.22 (29.25)	12.44 (10.12)	500:1	4.18 (2.16)	9.56 (8.55)	23.62 (26.80)
	250:1	2552 (3605)	289 (404)	34.38 (43.03)	250:1	4.10 (1.84)	7.26 (5.85)	13.85 (14.60)
	125:1	2960 (4184)	1632 (2304)	91.57 (124.8)	ND	ND	ND	ND
	62.5:1	1134 (1603)	156 (218)	22.67 (28.33)	ND	ND	ND	ND
<i>C. albicans</i> 200C	8000:1	6.39 (3.83)	8.21 (1.97)	11.40 (1.7)	8000:1	17.19 (9.35)	53.65 (46.32)	177.54 (192)
	4000:1*	5.88 (3.71)	7.09 (1.43)	9.52 (2.58)	4000:1	14.98 (7.10)	41.89 (33.82)	123.73 (129)
	2000:1	4.32 (2.80)	5.68 (1.02)	8.49 (2.81)	2000:1	12.69 (5.67)	35.55 (28.36)	106.57 (111)
	1000:1	5.52 (1.73)	6.93 (3.15)	8.82 (5.12)	1000:1*	10.21 (3.37)	28.13 (23.72)	87.74 (100)
	500:1	12.69 (11.3)	10.16 (8.43)	8.19 (6.20)	500:1	9.59 (4.83)	23.94 (19.46)	62.90 (65.20)
	250:1	5.40 (2.69)	5.81 (3.55)	6.33 (4.43)	250:1	8.79 (6.10)	20.67 (18.01)	49.53 (50.20)
<i>C. albicans</i> 201M	8000:1	3.23 (0.10)	4.27 (0.05)	5.63 (0.05)	8000:1	11.00 (6.08)	29.18 (17.37)	77.46 (49.30)
	4000:1*	3.14 (0.48)	3.89 (0.13)	4.88 (0.45)	4000:1	14.37 (9.01)	29.62 (18.84)	61.14 (39.20)
	2000:1	2.71 (0.30)	3.43 (0.18)	4.36 (0.06)	2000:1	13.04 (9.67)	26.39 (17.80)	53.50 (32.40)
	1000:1	2.26 (0.23)	2.91 (0.16)	3.74 (0.06)	1000:1*	11.28 (8.60)	21.87 (15.65)	42.35 (28.50)
	500:1	1.45 (0.35)	2.04 (0.36)	2.98 (0.45)	500:1	6.74 (5.01)	16.02 (11.91)	37.98 (28.40)
	250:1	3.53 (2.16)	3.59 (1.68)	3.66 (1.22)	250:1	4.89 (3.94)	11.73 (9.51)	28.39 (22.60)

*Represents equipotency ratios (ratios of MIC₅₀).

therapy and prevention of neonatal sepsis. Lactoferrin has been effective in animal models of infection and in polymicrobial infections *in vivo*. Clinical studies are needed to evaluate the potential efficacy of TLF in the treatment and prophylaxis of neonates against systemic infections to reduce mortality and morbidity.

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