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## Seminars in Fetal &amp; Neonatal Medicine

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## Lactoferrin: Antimicrobial activity and therapeutic potential

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## S U M M A R Y

## Keywords:

Lactoferrin  
Late-onset infection  
Microbiome  
Necrotising enterocolitis

Lactoferrin is a highly conserved protein from an evolutionary perspective, with a wide range of roles related to protection from infection and promotion of nutritional status. Infection, malnutrition and intestinal pathologies are key inter-related problems, represent important threats to survival and are associated with adverse long-term health outcomes after preterm birth. Lactoferrin is available as a commercial extract from bovine milk and offers potential as a therapeutic intervention for preterm infants modulating infections and intestinal pathologies. In this review we explore the structure, direct antimicrobial effects, modification of host immune function and gastrointestinal effects of lactoferrin. Current trial data are reviewed, and research priorities and challenges identified and discussed.

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## 1. Introduction

## 1.1. Late-onset infection and necrotising enterocolitis

Despite increased survival and improving long-term outcomes among preterm infants, the prevalence of necrotising enterocolitis (NEC) and late-onset sepsis (LOS) remains high. Combined, these major neonatal pathologies cause more late neonatal deaths than any other single cause.<sup>1</sup> Rates vary between neonatal units depending on case-mix and care practices, and correlate tightly with degree of prematurity. In developed countries up to 20% of very preterm ( $\leq 28$  weeks of gestation) or very low birthweight (VLBW,  $< 1500$  g) infants develop at least one episode of microbiologically confirmed or clinically strongly suspected LOS.<sup>2</sup> Significant adverse effects of infection mean that many clinicians prefer to treat if in doubt, but this increases antibiotic exposure.

Necrotising enterocolitis occurs in around 5–10% of very preterm infants, but is difficult to define robustly unless there is histological confirmation at surgery or at postmortem. Survivors risk longer-term complications such as short bowel syndrome, poor growth and impaired long-term cognitive outcome.<sup>3</sup> The aetiology of NEC is described as including the classic triad of enteral feeding, microbial colonisation and disruption to the integrity of the bowel wall, either

due to an ischaemic or inflammatory process. It is increasingly clear that NEC is not a single 'disease' but is likely to represent the end result of several interacting and modulating factors.<sup>4</sup>

## 1.2. Immunonutrition

Feeding very preterm infants with artificial formula rather than expressed maternal breast milk (EBM) increases the risk of NEC and LOS. EBM contains several immune-protective and growth factors, bioactive immune-modulatory cells and other 'immunonutrients' including amino acids, fatty acids, lysozyme, lactoferrin, minerals and metals such as zinc, and prebiotic oligosaccharides. Glutamine and arginine influence gut integrity and sepsis and several vitamins have key roles in antioxidant protection.<sup>3</sup> Lactoferrin has immunomodulatory properties and might modulate processes like NEC and LOS: higher levels in colostrum suggest that it might be a key component influencing a reduction in NEC and sepsis in breastfed preterm infants, although both diseases still occur in breastfed infants. A single controlled trial in neonates suggests that supplemental bovine lactoferrin may be beneficial in this population.<sup>5</sup>

## 2. Nutrition and the microbiome in preterm infants

## 2.1. The preterm microbiome

Emerging evidence suggests that both NEC and LOS are strongly related to patterns of microbial colonisation in the gut (referred to

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as the gut microbiome),<sup>6</sup> itself strongly influenced by management within neonatal intensive care units.<sup>4,7</sup> The non-human genetic material in our bowels outnumbers our own DNA by more than 10-fold and includes bacterial, viral, and archaeal elements. Enteric organisms contribute to a wide range of essential functions, including immunomodulation through education of gut-associated lymphoid tissue, induction of tolerance to commensal bacteria, and maintenance of endothelial tight junctions.<sup>8,9</sup> Microbial community perturbations (dysbioses) may initiate complex processes clinically recognised as NEC or LOS<sup>4,10–12</sup>; many bacteria cultured in LOS are of bowel origin (Table 1).<sup>2</sup> The exploration of roles of all microbial taxa in this has been revolutionized through the application of next-generation sequencing techniques.<sup>13,14</sup>

## 2.2. Modifying the microbiome to prevent NEC and LOS

Recent research efforts have investigated the potential for modulation of the preterm microbiome that might reduce NEC and LOS. The interactions involved are likely to be complex and modulated through exposure to various risk factors (e.g. formula milk feeding, antibiotic use, immune-nutrient deficiency) and interactions between immunotype, enterotype and phenotype (Fig. 1).<sup>4</sup> These risks will be affected by both antenatal and postnatal care practices, infection control, and dietary interventions, etc.

## 2.3. Probiotics and lactoferrin

Several trials of probiotic supplementation and meta-analyses suggest significant reductions in the risk of NEC, but, given the apparent close relationship between bacterial translocation in NEC and LOS, no overall reduction in LOS has been reported.<sup>15</sup>

Lactoferrin is a key mammalian protein with a bewildering array of functions still being elucidated more than 50 years after the initial 'discovery' in 1960.<sup>16</sup> Excellent reviews exist<sup>17</sup>; here we provide a brief overview to enable better understanding of functional properties and how this is of relevance to NEC and LOS in the preterm population.

## 3. Lactoferrin structure

Lactoferrin is secreted by epithelial cells into exocrine fluids: seminal fluid, pancreatic exocrine secretions, tears, saliva, uterine secretions, and milk. Neutrophils also secrete lactoferrin locally at sites of inflammation. Control of production and regulation in various body fluids is complex, dependent on local hormonal feedback mechanisms, and/or controlled by progressive cell differentiation or death. Levels vary greatly, with the highest concentration in mammalian milk (7 g/l human colostrum, 1 g/l mature human milk and 0.4 mg/l in 'normal' human plasma, increasing 5000-fold in infection). Milk from mothers who deliver preterm may differ from those delivering at term and contain less human

**Table 1**  
Principal pathogens responsible for late-onset sepsis (LOS) in UK.

Organism	% of LOS
Enterobacter <sup>a</sup>	21
Enterococcus <sup>a</sup>	16
Meticillin-susceptible <i>Staphylococcus aureus</i>	16
<i>Escherichia coli</i> <sup>a</sup>	13
<i>Candida</i> spp. <sup>a</sup>	9
Group B streptococcus	8
<i>Pseudomonas</i> spp.	5
Other	12

Data from Vergnano et al.<sup>2</sup>

<sup>a</sup> Denotes organism commonly originating from bowel.

lactoferrin.<sup>18,19</sup> Lower concentrations in preterm milk and the delayed establishment of milk feeding<sup>20</sup> mean that preterm infants receive relatively small amounts of human lactoferrin in their first weeks when they are most at risk. Formula milk receipt also exposes these infants to much lower levels of lactoferrin than EBM (Fig. 2).

### 3.1. Molecular structure

The lactoferrin gene (chromosome 3)<sup>21</sup> codes for production of lactoferrin or delta-lactoferrin (nucleocytoplasmic form) dependent on promoter use.<sup>22</sup> Knockout mice exist with normal iron homeostasis mechanisms.<sup>23</sup> One polypeptide chain is folded to produce a similar structure to transferrin – two lobes linked by a small peptide chain (Fig. 3). The lobes (N and C) each carry a glycosylation site and an iron binding site and configure differently when carrying iron or glycosylated. These structural alterations explain differences in function between apolactoferrin (iron free) and hololactoferrin (iron rich). Key peptide clusters of lactoferrin are known to confer the specific functional properties of iron sequestration, nuclear targeting, lipopolysaccharide (LPS) binding, modulation of inflammatory response, antimicrobial activity, and stimulation of apoptosis, etc.

Lactoferrin is subject to proteolysis and some important functions are delivered by the cleaved fractions: lactoferricin (25 amino acids long), structurally altered to configure as a beta-sheet, has better contact with microbes and is responsible for many 'direct' antimicrobial effects of lactoferrin.

### 3.2. Species variation and specific functions

Although lactoferrin molecules from different species are highly homologous, even small differences in peptide cluster structures can affect function. Some activities of human lactoferrin are shared by bovine lactoferrin, but not all. For example, the pH at which lactoferrin releases iron differs among species (pH 2 in humans, pH 4 in cows and pH 6 in camels) and is likely to reflect different evolutionary pressures (see Table 2 in Legrand<sup>17</sup>). In vitro, bovine lactoferrin promotes human intestinal cell proliferation and differentiation, and increases the expression of transforming growth factors and cytokines.<sup>24</sup>

These mechanisms may modulate tight junctions and the inflammatory cascade, and thus may be potential key mechanisms in prevention of NEC and gut-related LOS.

### 3.3. Synthetic lactoferrin

Talactoferrin is a recombinant human lactoferrin produced commercially using aspergillus. Its main use has been for cancer treatment, but in-vitro activity has been shown against candida and coagulase-negative staphylococcus (CONS).<sup>25</sup> No neonatal trials are published.

## 4. Lactoferrin function in relation to NEC and sepsis

### 4.1. 'Direct' antimicrobial effects

- Iron sequestration – since many bacteria require iron to function, the antibacterial properties of lactoferrin were initially attributed to its capacity to bind free iron. Human lactoferrin is normally <10% saturated with iron: states that increase this are associated with ill health, such as cystic fibrosis. However, some bacteria can use the iron in lactoferrin, and other bacteria (including *Neisseria* spp., *Haemophilus* spp., *Escherichia coli*, and *Pseudomonas* spp.) are unaffected by

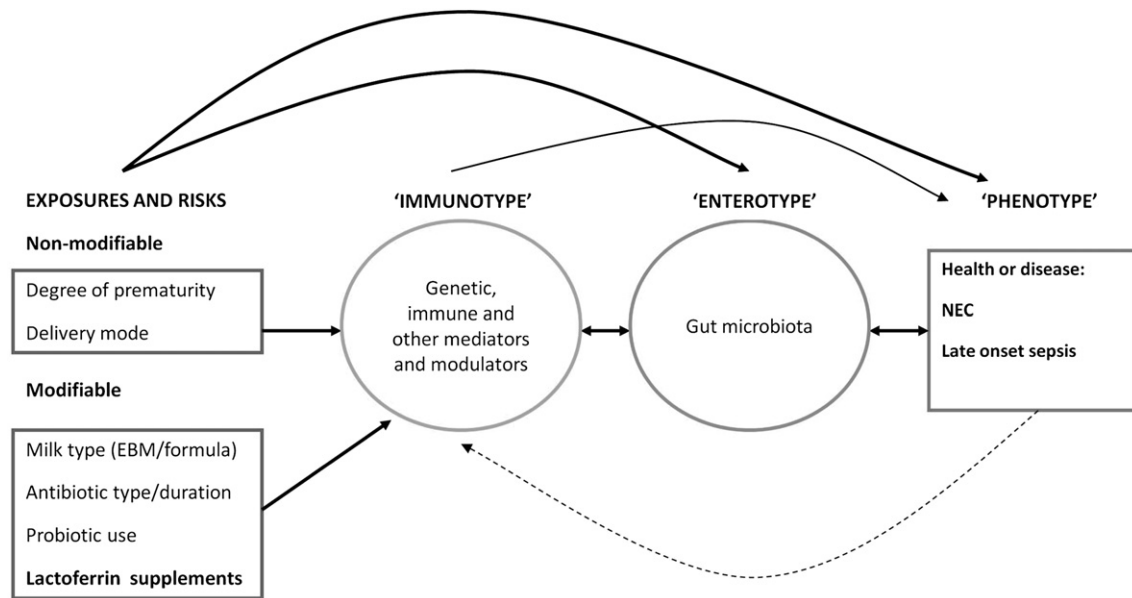


Fig. 1. Interactions between risk factors and health or disease phenotype. EBM, expressed maternal breast milk; NEC, necrotising enterocolitis.

environmental iron levels by producing iron-chelating proteins called siderophores.<sup>26</sup>

- Destabilisation of micro-organism cell membrane: the mechanism appears to differ between Gram-negative and Gram-positive organisms. For Gram-negatives, lactoferrin binds to porins present on the surface causing LPS release, and increasing bacterial fragility.<sup>27</sup> The calcium chelation activity of lactoferrin also induces LPS release.<sup>28</sup> Gram-positive bacterial membranes are disrupted by cationic residues and hydrophobic residues in the N-terminus. Fungal wall disruption appears dependent on extracellular cation concentration: in-vivo

lactoferrin effects on different fungal species differ, being most effective for *Candida tropicalis*, and least effective for *Candida glabrata*.

- Modification of micro-organism motility: glycosylated lactoferrin can bind to bacterial adhesion sites on bacteria and host cells, thus preventing bacterial adhesion – different effects are seen with the recombinant form, which has different glycosylation patterns.
- Lactoferrin is able to bind onto receptors on host cells that viral and bacterial pathogens use to gain entry, such as glycosaminoglycans (GAGs). By competitive inhibition lactoferrin can

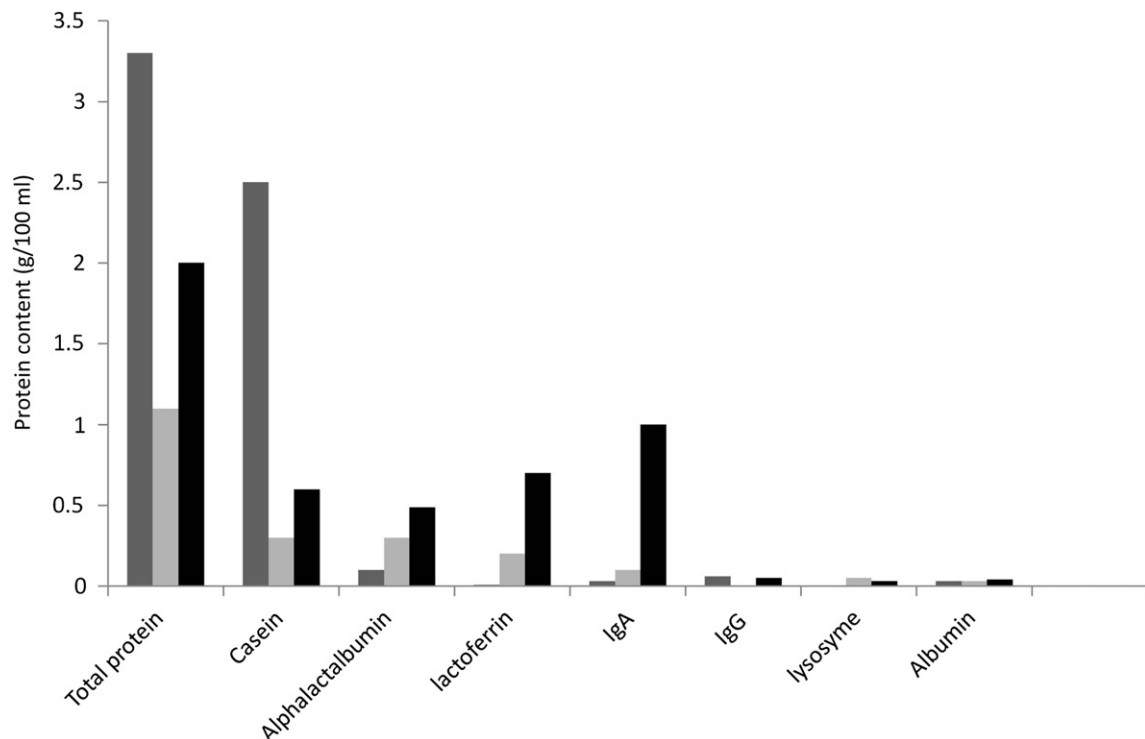


Fig. 2. Protein content of cows' milk, human colostrum and mature human milk (g/100 ml). ■, cows' milk; ■, mature human; ■, human colostrum.

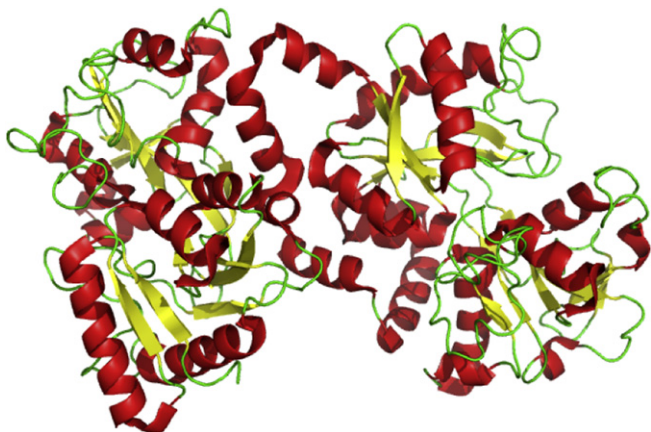


Fig. 3. Structure of lactoferrin.

then reduce endocytosis of the micro-organism into host cells where they can evade the immune response; this mechanism is used by some strains of *E. coli* that are enteroinvasive, and *Staphylococcus aureus*.<sup>28</sup> This mechanism also appears to confer protection against viral pathogens. Reduced viral loads in neonatal rats infected with cytomegalovirus have been shown after bovine lactoferrin pretreatment, but in human preterm infants maternal milk lactoferrin levels did not influence viral transmission rates of hepatitis C virus.<sup>29</sup>

- Disruption of biofilms – iron-binding activity of lactoferrin leads bacteria to ‘move’ in search of iron, thus disrupting biofilms which depend on static bacterial presence.<sup>29</sup> The iron-rich mucus of those with cystic fibrosis promotes *Pseudomonas* and *Burkholderia* spp., both of which exert virulence through biofilm formation, and the protective effect of lactoferrin appears lost in this iron-rich environment.
- Modification of virulence factors: many bacteria produce protein virulence factors which lactoferrin may degrade through proteolysis. Such proteolysis induced by the N-lobe of lactoferrin has been demonstrated in vitro for *H. influenza*, *Shigella* spp. and *E. coli*.<sup>30</sup>

#### 4.2. Modification of host immune function

Lactoferrin, like many immunomodulators, plays a complex role in the immune cascade and can both up- and downregulate the endogenous inflammatory response, possessing pro- and anti-inflammatory properties.

- Downregulatory mechanisms:
  - Lactoferrin binds to the lipid portion of LPS, neutralising the ability of LPS to interact with toll-like receptor 4; limits initiation of the inflammatory cascade; enhances interleukin-10 production; and reduces tumour necrosis factor- $\alpha$  production, thereby encouraging Th2 (anti-inflammatory)-like activity.
  - Reduced production of reactive oxygen species such as oxygen ions and peroxides: these require free iron for synthesis, so chelation of iron by apolactoferrin reduces production, ameliorating the inflammatory response.
  - Cell recruitment downregulation – cell migration to the site of inflammation is necessary for the promotion of the inflammatory cascade. Lactoferrin interference appears mediated through fibroblast gene expression, modulating protein production and altering the extracellular matrix, thus modulating motility of immune cells.

- Upregulatory mechanisms:
  - Lactoferrin promotes maturation of T-lymphocytes: under different host conditions promotion of either Th1 (pro-inflammatory) or Th2 (anti-inflammatory) cytokine profiles occurs.<sup>31</sup>
  - Orally ingested lactoferrin recruits and activates antigen-presenting cells, promoting the inflammatory cascade.<sup>32</sup>
  - In chronic viraemia, oral lactoferrin initiates interleukin-18 production, promoting a Th1 cytokine profile in peripheral blood.<sup>33</sup>
  - Oral bovine lactoferrin stimulates gut-associated immune functions by production of interleukin-18, type I interferons and increased natural killer cell activity.<sup>34</sup>

#### 4.3. Direct effect on gastrointestinal development

Enteral lactoferrin has a direct effect on enterocyte growth and differentiation in vitro and may exert a protective effect through direct action on the enterocytes. Bullen et al.<sup>35</sup> demonstrated that in-vivo effects on bacterial proliferation could also protect neonatal guinea-pig gut colonisation with *E. coli*. Lactoferrin affects bacterial, fungal and viral elements though not all mechanisms are relevant to all: iron sequestration affects bacteria only, and membrane disruption affects bacteria and fungi, but not viruses. Some processes culminate in pathogen death (bactericidal) whereas others are bacteriostatic. Ecologically it seems likely that stasis or cidal activity might liberate a niche resulting in altered microbiomic conditions that affect NEC or LOS occurrence.

The complexities involved in lactoferrin interaction with inflammatory processes and the challenges of extrapolation of in-vitro studies to clinical situations emphasise the need for carefully designed and conducted controlled trials.

#### 5. Lactoferrin function: relevance to other neonatal conditions

Although the current focus is the potential role of lactoferrin in preventing LOS and NEC, it may also prove beneficial to preterm infants in other ways. Whereas lactoferrin does cross the blood–brain barrier it may play a role in neurodegenerative conditions and a direct neuroprotective effect is possible.

Lactoferrin is a potent anabolic factor affecting osteocytes, stimulates osteoblast proliferation, enhances thymidine incorporation into osteocytes, and reduces apoptosis of osteoblasts.<sup>36</sup> Talactoferrin modulates neutrophil-induced gastrointestinal injury mediated by non-steroidal anti-inflammatory drugs<sup>37</sup>: this may be relevant to preterm infants treated for patent ductus arteriosus. Serum and stool levels of lactoferrin have been explored as a potential indicator of NEC development, but currently show little clinical promise due to wide variance in levels detected.<sup>38</sup>

Intriguingly, lactoferrin delivered vaginally may prevent preterm labour via iron modulation and an anti-inflammatory effect.<sup>39</sup> Normalisation of vaginal flora in at-risk women after oral administration of lactoferrin has also been reported.<sup>40</sup> Beneficial effects have also been demonstrated in vitro on rotavirus attachment and replication<sup>41</sup> and amoebal and giardial structure. Enteric bacteria affected by lactoferrin include those that contribute to significant diarrhoeal death across the globe: *Shigella* spp., *Salmonella* spp. and *E. coli*,<sup>42</sup> and have recently been reviewed by Ochoa.<sup>43</sup> Effects on rotavirus severity,<sup>44</sup> symptom duration<sup>45</sup> and growth<sup>46</sup> may all be relevant for neonates. Impacts on biofilm formation and the importance of these for central venous catheter infections merits further exploration: in contact lenses coated with lactoferrin 50% fewer bacteria were seen.<sup>47</sup> Because the role of viruses and archae



in bowel pathology remains to be determined, the effects of lactoferrin on herpes simplex virus, cytomegalovirus, human immunodeficiency virus and rotavirus<sup>48</sup> may be of relevance.

## 6. Lactoferrin supplementation in preterm infants

### 6.1. Evidence from clinical trials

Human milk-fed infants have lower rates of NEC and LOS,<sup>49,50</sup> and, along with mechanistic knowledge of lactoferrin, led to the first neonatal trial of supplemental bovine lactoferrin in Italy.<sup>5</sup> A subsequent small ( $n = 80$ ) study undertaken in Canada remains unreported to date (LACUNA: LACToferrin Use in Neonates ISRCTN66482337). The Italian controlled trial randomised 472 infants in 11 neonatal units to receipt of either 100 mg/day of bovine lactoferrin daily or 5% dextrose. The study design also included a group that received both bovine lactoferrin and a probiotic *Lactobacillus rhamnosus* GG (LGG). The primary outcome measure was the incidence of first episode of LOS defined as isolation of any pathogen in blood, cerebrospinal fluid or peritoneal fluid. Bovine lactoferrin (or placebo) was started on day 3 and administered even if milk feeds had not commenced for 30 days (birth weight 1000–1500 g) or 45 days (birth weight <1000 g). Infants were excluded if they had received prophylaxis against fungus with fluconazole. Overall results showed that both bovine lactoferrin alone and bovine lactoferrin plus LGG reduced the total burden of LOS. The major effect was achieved by reducing fungal LOS, but reductions in Gram-positive infections were also seen. Stratification by birth-weight showed that those infants <1000 g benefited most. This may be due to the dose of bovine lactoferrin not being adjusted for weight, or that the background risk in these infants was lower. This initial publication was not powered to assess an effect on NEC, although a lower incidence was seen in the bovine lactoferrin plus LGG group. Later data presented thus far in abstract form at a meeting of the Pediatric Academic Societies meeting (PAS Denver 2011) of an overlapping population of 825 infants suggests a reduction in risk of NEC (> stage 2) of relative risk = 0.35 [95% confidence interval (CI): 0.13–0.99;  $P = 0.04$ ]. These data await publication following peer-review.

### 6.2. Challenges of existing data

This first clinical study of bovine lactoferrin is of major clinical importance, with an odds ratio for LOS of 0.32; 95% (CI: 0.14–0.77) for what is a relatively cheap and readily available orally delivered intervention. As with probiotics, it is an intervention that could also be available in resource-poor settings. However, the study has several important considerations that require addressing before lactoferrin use could be considered as a standard of care. The Italian units appear different in comparison with most UK units, having a relatively high incidence of LOS of fungal origin; much of the benefit was in reducing fungal sepsis. Many UK units and others in both Europe and Australia (but perhaps not in the USA) have much lower rates of fungal infection either naturally or due to their use of prophylactic fluconazole. The exclusion of infants receiving fluconazole from the study is of interest, since fluconazole had been shown by many of the same Italian units to reduce the incidence of fungal infection.

The lack of benefit from bovine lactoferrin in the heavier babies also raises questions about dosage. This was based on the mean human lactoferrin intake that VLBW neonates ingest with mother's fresh milk in the first 2 weeks of life (30–150 mg/d), but no adjustment was made for birth weight; thus a 500 g baby received three times as much per kilo as a 1500 g baby. Optimum dosing may not have been delivered to bigger babies. Blinding in any study is

challenging, but especially so with orally administered nutritional supplements. In the study by Manzoni et al.<sup>5</sup> the placebo was 5% dextrose: this may have characteristics different from those of a protein/peptide solution. In addition, bovine lactoferrin will tend to bind ferric ions, and some preparations may have a slight red or brown discoloration. But it is debatable whether inadequate blinding would have any effect on primary trial outcomes.

The Italian trial was planned with a power of 80% to detect a reduction in the risk of LOS of any origin from 18% to 6%, and may be underpowered for many secondary outcomes. The combination group of bovine lactoferrin + LGG is of great interest as probiotic prophylaxis becomes increasingly widespread, but the study was relatively underpowered to detect differences in LOS between the bovine lactoferrin group and the bovine lactoferrin + LGG group because of low incidence overall in these two groups. The effects of combining bovine lactoferrin and probiotics may differ if different probiotics or probiotic combinations are used, and may act in a synergistic or antagonistic fashion. As with any 'first' study, the findings require replication in other settings.

Processing and sterilization of commercial additive bovine lactoferrin and manipulation of feeds within neonatal units may both also have effects on function and therefore efficacy. Pasteurization of bovine milk is known to reduce bovine lactoferrin 'content' by 65% but other manipulations of milk undertaken within units may also affect efficacy such as freezing, thawing, adding fortifier, electrolytes, and vitamins, etc.<sup>51</sup> These have not been explored.

## 7. Research: challenges and priorities

Supplemental bovine lactoferrin appears to hold promise as an intervention that might benefit preterm infants by reducing mortality and serious morbidity, improving long-term quality of life in survivors and savings in healthcare costs. The importance of NEC and LOS and the apparent effect size of bovine lactoferrin treatment in this population make it tempting to consider introducing this as a prophylactic intervention now. However, the existing data leave questions unanswered and are insufficient without replication in further large-scale randomised controlled trials. If bovine lactoferrin is really to have a significant global impact on neonatal LOS, efficacy must be demonstrated in other populations and resource settings. Additionally, we need to know whether it is still efficacious in the face of other practices that might interact such as other milk or feed additives (e.g. breast milk fortifier) and the use of other promising enteral interventions such as probiotics. It remains unclear what effect size bovine lactoferrin may have in units with either low fungal incidence or where the use of prophylactic fluconazole is common. It is also unclear whether bovine lactoferrin may work more effectively when dosing is per kg body weight. Further studies may be required to determine the optimal dose.

It is possible that an intervention that improves early outcomes will worsen later ones such as neurodevelopmental impairment. Where trials show a reduction in key morbidities likely to affect long-term cognition (such as reduction in sepsis or NEC) it might seem reasonable to power studies on a reduction in their incidence, but there still may be trade-offs. In all of these areas of debate, many would now agree that clinicians have a duty to involve parents (and their advocates) in the planning of trials at all stages, from choice of intervention and outcome, through to conduct of the trial. Parental perspectives on relative 'trade-offs' may need to be sought where a trial intervention reduces one morbidity at the cost of another. The ideal trial then would assess both short- and long-term outcomes, and include a health economic analysis, but funding such trials is often prohibitively expensive.

Despite these challenges it seems clear that a large, pragmatic, multicentre, blinded, randomised, controlled trial of supplemental

bovine lactoferrin is a research priority and several groups are now working towards delivering this aim. Among these trials is the UK trial run by the ELFIN (Enteral LactoFerrin In Neonates) collaborators group.<sup>52</sup> The ELFIN trial is powered on a primary outcome of LOS based on the incidence gathered by the NEONIN studies, which collate data from a diverse mix of UK neonatal units. Some of the units use antifungal prophylaxis and some are likely to be using probiotics. It aims to recruit 2200 infants with birth weight <1500 g and will be able to detect with 90% power a 25% relative risk reduction. The study design allows for formula or breast milk receipt, or mixed feeding. The dose delivered will be based on a per kg weight basis, thus ensuring that larger babies also receive a similar dose. This is also more in keeping with the levels ingested by enterally fed term infants.

## 8. Summary

Lactoferrin is a complex molecule that can be extracted from cow's milk in large quantities and be produced in a recombinant human form. Although highly homologous, these forms differ functionally, potentially in key mechanistic actions. Bovine lactoferrin is the most researched and has been shown safe and promising in preterm neonates. It appears to hold significant potential to reduce major morbidities in preterm infants of worldwide importance, but further confirmatory trials are needed.

### Practice points

- Human colostrum contains much more lactoferrin than bovine milk, emphasising the importance of ensuring that all preterm infants are able to receive mothers' own breast milk.
- Late-onset infection and necrotizing enterocolitis are major causes of mortality and serious morbidity and are both influenced by interactions with the preterm microbiome.
- Lactoferrin exerts direct antimicrobial, host immunomodulatory, and direct and indirect gastrointestinal effects.

### Research directions

- Neonatal studies must focus on goals that matter to parents and infants: reducing mortality and serious morbidity and improving long-term quality of life in survivors.
- Appropriately designed and powered trials of supplemental lactoferrin are needed to assess whether it offers therapeutic benefit by reducing LOS and/or NEC.
- An improved understanding of the underpinning mechanisms of microbiomic, gastrointestinal and immunomodulatory role of lactoferrin will enable refinement of dosage schedules, populations that might benefit, and alternative interventional strategies.

### Conflict of interest statement

None declared.

### Funding sources

None.

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