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Lactoferrin and prematurity: a promising milk protein?

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Abstract

Lactoferrin (Lf) is the major whey protein in milk with multiple beneficial health effects, including direct anti-microbial activities, anti-inflammatory effects and iron transporter. Iron homeostasis in the foetus, the neonate and preterm infant is crucial for adequate growth and development. Lf oral supplementation in human preterm infants has been shown to reduce the incidence of sepsis and necrotizing enterocolitis. In preclinical models of antenatal stress and perinatal brain injury bovine Lf protected the developing brain from neuronal loss, improved connectivity, increased neurotrophic factors and decreased inflammation. It also supported brain development and cognition. Further, Lf could prevent preterm delivery, through a reduction of pro-inflammatory factors and inhibition of premature cervix maturation. We review here the latest research on lactoferrin in the field of neonatology.

Introduction

Despite considerable progress in neonatal medicine and an improved survival rate of children born prematurely, the incidence of preterm births has increased in most countries (Harrison and Goldenberg 2015). These infants are at risk of infections, inflammation, oxidative stress that can lead to serious disease such as sepsis, bronchopulmonary dysplasia, necrotising enterocolitis, and preterm encephalopathy or periventricular leukomalacia (Frey and Klebanoff 2016) all risk factors for later neurodevelopmental disabilities. Infection/inflammation in the mother and/or foetus is not only a risk factor for preterm birth but also one of the main cause of cerebral white and grey matter injuries in the preterm infant as well as aggravating factor for other perinatal cerebral insults such as hypoxia/ischemia (Volpe 2009). These later becoming a leading cause of long term neurodevelopmental disabilities including cerebral palsy, learning impairment, visual and hearing disorders, language difficulties and also psychiatric illnesses at adult age (Back and Miller 2014; Salmaso et al. 2014). In Europe and other developed countries, the incidences of premature birth range from 6 to 12% with an increasing trend over the last decade. High-risk pregnancies with prematurity are a large burden to the society due to the high morbidity, social and economic

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costs associated, both for the mother and the child (Bhutta et al. 2014; Hoyert et al. 2006; Walker et al. 2007).

On the other hand, breast milk has recognised beneficial effects in term and preterm infants, including decreased rates of infection and NEC and improved cognitive and behaviour skills (Lucas and Cole 1990; Meinzen-Derr et al. 2009; Quigley et al. 2007; Sullivan et al. 2010; Vohr et al. 2007; Vohr et al. 2006). The protective effects of human milk are due to the multiple factors transmitted through milk, including secretory antibodies, oligosaccharides, glycoconjugates, lactoferrin (Lf), lysozyme, leukocytes, cytokines and other factors produced by the mother's acquired and innate immune systems (Ballard and Morrow 2013; Walker 2010). Lf is the second most abundant protein in human milk.

Lactoferrin (Lf) is a physiological compound produced by exocrine glands and released at a high level in colostrum and maternal milk (Ronayne de Ferrer et al. 2000). It plays numerous biological and beneficial functions such as iron absorption, anti-inflammatory action, immunomodulator, antioxidant, host defence mechanism and anti-cancer agent (Sandomirsky et al. 2003; Satue-Gracia et al. 2000; Wong et al. 1998). High levels are found in human milk (Ronayne de Ferrer et al. 2000) but not in most of formula milk resulting in a higher level of iron-induced oxidation products in preterms formula fed (Raghuvver et al. 2002). Free radical injury plays a significant role in several prematurity related diseases: brain injury, necrotising enterocolitis, retinopathy, bronchopulmonary dysplasia. (Kelly 1993). The role of Lf as a potential protectant in preterm infants and its effects in prematurity related translational research are reviewed here.

Lactoferrin and neonatal infection

Infections are one of the main causes of morbidity and mortality in preterm neonates. Neonatal sepsis is responsible for 13% of all neonatal mortality and 42% of deaths in the first week of life (Lawn et al. 2005; Liu et al. 2012). Rates of infection increase with decreases in birth weight. Very low birth weight infants (VLBW) (<1500g) have the highest rates of sepsis. Premature infants with neonatal infections have prolonged hospital stays, higher mortality, impaired growth and are more likely to have adverse neurodevelopmental outcomes at follow up compared with those uninfected (Adams-Chapman and Stoll 2006; Bassler et al. 2009; Stoll et al. 2002; Stoll et al. 2004).

In addition to sepsis, premature infants are at higher risk for developing necrotizing enterocolitis (NEC). Although NEC occurs only in approximately 7% of VLBW infants, it is the most lethal gastrointestinal disease in the neonatal population. Consistent risk factors are prematurity and low birth weight. However, the pathogenesis of NEC is not entirely clear, but intestinal immaturity, enteral feedings (especially infant formula feeding), bacterial colonization, and inflammation all play a role (Lin and Stoll 2006; Neu and Walker 2011). The estimated rate of death associated with NEC is 20 to 30%, with the highest rate among infants requiring surgery. Infants with a history of NEC have delayed neurodevelopmental outcomes; they have a 25% chance of microcephaly (Hintz et al. 2005; Lin and Stoll 2006). The financial cost of NEC is substantial; the total annual estimated cost of caring for

affected infants in the United States is between \$500 million and \$1 billion (Neu and Walker 2011).

The increased risk of preterm infants to infections is, in part, due to the immaturity of their immune systems. All aspects of the immune system, including primary (bone marrow), thymus, and secondary lymphoid organs, progressively develop during foetal life, thus preterm infants are born with a less mature immune system than infants at term (Melville and Moss 2013). This immature immunity can result in long-term immune dysfunction. In addition, the immune changes that occur with NEC and sepsis can also result in immune dysfunction, including loss of dendritic cells, decrease in T and B cells, and shift towards T cell helper type 2 immunity. These changes can last months after recovery from disease (Delano et al. 2007).

We hypothesize that Lf is the major factor responsible for the protective effects of breast milk-decreased rates of infection and NEC, improved neurodevelopment and better immune responses due to its antimicrobial, anti-inflammatory and immunomodulatory properties (Figure 1) (Baker and Baker 2012; Brock 2012; Vogel 2012).

Pre-clinical studies

Antimicrobial effect of lactoferrin—In vitro and in vivo studies have shown a protective effect of human and bovine Lf against infections. Lf antimicrobial mechanisms are multiple: (1) Iron sequestration, which is essential for bacterial growth. (2) Destabilization of microorganism cell membrane: Lf binds to the lipid A portion of lipopolysaccharide (LPS) on the cell surface of Gram-negative bacteria, disrupting the bacterial cell membrane; it also has anti-lipoteichoic acid (Gram positive organisms) and anti-Candida cell wall activities. (3) Binding to viral and bacterial host cell receptors, decreasing the ability of pathogens to adhere or to invade mammalian cells. (4) Modification of virulence factors, by binding or degrading specific virulence proteins. (5) Inhibition and disruption of biofilm formation. (6) Bactericidal activity by lactoferricin, a N terminus peptide fragment of bovine Lf. (7) Intestinal flora modulation. (8) Promotion of intestinal cell proliferation, differentiation and maturation (Embleton et al. 2013; Ochoa and Cleary 2009; Vogel 2012).

In animal models, bovine Lf has protected mice from a lethal dose of parenterally administered *E. coli* (Zagulski et al. 1989) and against endotoxin-induced lethal shock in piglets (Lee et al. 1998). Human Lf neutralizes endotoxin (Zhang et al. 1999) and protects rats from gut-related *E. coli* systemic infections (Edde et al. 2001). Oral Lf enhances clearance of both *E. coli* and *S. aureus* injected IV into mice (Artym et al. 2004a).

Anti-inflammatory and immunomodulatory properties of lactoferrin—Apart from a possible role in modulating iron homeostasis during inflammation, Lf may directly regulate the inflammatory response itself. Through binding of Lf to bacterial LPS there is a reduction of LPS-mediated upregulation of inflammatory cytokines. Further, Lf sequesters “free” iron at inflammatory foci, thus preventing catalysis of the production of damaging free radicals (Vogel 2012). These anti-inflammatory effects of Lf are probably initiated following release of Lf from neutrophils. Lf downregulates pro-inflammatory cytokines in

intestinal epithelial cells infected with invasive and noninvasive *E. coli* strains, which may represent an important natural mechanism in regulating epithelial cell responses to pathogenic bacteria and in limiting cell damage and the spread of infection (Berlutti et al. 2006). Much of the impact on extraintestinal manifestations of infections is likely to be related to immunomodulatory effects of oral Lf. Artym (Artym et al. 2004b; Artym et al. 2005; Artym et al. 2003) showed that in mice oral bovine Lf upregulates cellular and humoral immune responses. In summary, Lf reduces inflammation by decreasing production of tumor necrosis factor alpha and other proinflammatory molecules, and by regulating the immune response, protecting against severe inflammation related to infection and septic shock (Berlutti et al. 2006).

Recent animal models showed a protective effect of Lf against NEC development by modulating the gut microbiome. Transgenic milk containing recombinant human Lf enhanced the intestinal flora in piglets (the gut microflora had more diversity), as measured by 16S rDNA sequence analysis, compared to the control group, as well as higher concentrations of Bifidobacterium and Lactobacillus in the intestine (Hu, 2012). In a neonatal rat model of NEC, bovine Lf significantly reduced NEC incidence; the protective effect was associated with increased density of mucin-producing goblet cells in the terminal ileum and changed ileal morphology (Wittke 2013). In a piglet model of NEC Lf had positive effects: increased cell proliferation via extracellular signal-regulated kinase, limited IL-8 secretion and prevented NF- κ B and hypoxia-inducible factor-1 α activation, suggesting strong anti-inflammatory effects (Nguyen et al. 2014).

Clinical studies

Several clinical studies have been conducted in children to demonstrate the effect of Lf on different clinical outcomes, including: (1) iron metabolisms and anemia, (2) fecal flora, (3) enteric infections, (4) common pediatric illnesses, (5) immunomodulation in HIV children, and (6) neonatal sepsis and NEC (Ochoa et al. 2012). The efficacy of each trial has been variable; however, an important finding of all trials has been the safety of the intervention. No adverse events have been reported with the use of bovine Lf, human Lf or recombinant human Lf (Ochoa et al. 2012). Protection against neonatal infections is the most likely relevant activity of Lf in infants.

Currently, there are 11 registered clinical trials of Lf for prevention of neonatal sepsis (Turin, 2014). Five published clinical studies demonstrated a protective effect against sepsis and NEC (Akin et al. 2014; Kaur and Gathwala 2015; Manzoni et al. 2014; Manzoni et al. 2009; Ochoa et al. 2015), which has been reviewed in a recent meta-analysis (Pammi and Abrams 2015). Larger trials are underway to confirm the findings of these initial studies.

The first study testing Lf for prevention on of sepsis in neonates was conducted by Manzoni et al. in Italy (Manzoni et al. 2009). They randomly assigned 472 VLBW infants to receive orally administered bovine Lf (bLf) (100mg/day, LF100, Dicofarm SpA, Rome, Italy), bLf plus the probiotic *Lactobacillus rhamnosus* GG (bLf+LGG), or placebo for 30 days. The incidence of sepsis was significantly lower in the bLf and bLf+LGG groups compared with the placebo group (5.9% and 4.6% vs. 17.3%), with a risk ratio (RR, which is the risk of sepsis in the treatment group compared with the risk of sepsis in the control group) of 0.34,

95% confidence interval (CI) (0.17–0.70), $p=0.002$ for bLf vs. placebo. Death from sepsis also was reduced in both treatment groups compared with placebo (0% and 0.7% vs. 4.8%). Later, in a secondary analysis, the authors found a significant decrease in invasive fungal infections in both bLf groups (0.7 % with bLf and 2.0 % with bLf + LGG vs. 7.7 % with placebo, $p<0.05$) (Manzoni et al. 2012).

In a follow-up study, Manzoni evaluated prevention of NEC in 743 VLBW infants. The incidence of death and/or NEC was significantly lower in both treatment groups (4.0%, with bLf and 3.8% with bLf+LGG vs. 10.1% with placebo), with a RR 0.39, 95%CI (0.19–0.80), $p=0.008$ for bLf vs. placebo (Manzoni et al. 2014).

In a small study in Turkey conducted in 50 VLBW infants or born before 32 weeks (Akin et al. 2014) the authors reported a significant decrease in the rate of late onset sepsis (4.4 vs. 17.3/1,000 patient days, $p=0.007$) with bovine Lf treatment, at a dose of 200mg/day (LF100, Dicofarm SpA, Rome, Italy). The prevalence of NEC Bell stage 2 was 20% in the control group vs. 0 in the Lf group ($p<0.05$) (Bell Stage 1 is suspected, 2 is proven and 3 advanced NEC). However, this was a per-protocol analysis, since three patients in the Lf group were excluded from the analysis because they developed sepsis before the first dose of the study intervention. The per-protocol analysis, which includes only those patients who completed the treatment originally allocated, could lead to bias. Exclusion of patients with sepsis in the LF group will decrease the rate of sepsis in this group. Ideally, the analysis of treatment groups should include all patients as originally allocated after randomization (Intention-to-treat analysis).

Our group in Peru, recently reported the results of a pilot study in 190 infants with a birth weight $<2,500$ g, who were randomized to receive bovine Lf (Tatua Co-operative Dairy Co, Ltd., Morrinsville, New Zealand) 200mg/Kg/day in 3 divided doses each day or placebo (maltodextrine) for 4 weeks. In the intention-to-treat analysis, the cumulative sepsis incidence in the Lf group was 12.6% vs. 22.1% in the placebo group, with a crude RR of 0.57, 95% CI (0.30–1.09). Among VLBW neonates the sepsis rates in the Lf group was 20.0% vs. 37.5% in the placebo group, a 46% reduction in sepsis. The RR adjusted for birth weight category was 0.57, 95% CI (0.30–1.07). In a secondary exploratory model, using time since the start of treatment, Lf achieved significance (Ochoa et al. 2015).

A recent study from India in 132 low birth weight infants ($<2,000$ g), conducted by Kaur and Gathwala (Kaur and Gathwala 2015), report a reduction in the prevalence of culture proven late onset sepsis with bovine Lf (3.2% vs. 13.4%), RR 0.211, 95%CI (0.044–1.019) (Kaur, 2015). However, this publication has several limitations. First, two patients from the Lf group were excluded from the analysis after randomization because they developed early onset sepsis; therefore is not an intention-to-treat analysis. As previously described, the analysis of treatment groups should include all patients as originally allocated after randomization; the exclusion of patients after randomization introduces bias. Second, the authors do not report the type or brand of bovine Lf used. Third, the dose of Lf was variable between 80 and 142 mg/Kg/day, with higher doses for the infants with higher birthweight. Finally, based on the dose description, seems they enrolled infants with a birthweight between 1,000 and 2,000 g, which is not clear in the description of the population enrolled.

In summary, previous clinical studies, despite the small sample size of some, have demonstrated a protective effect against neonatal infections supported by a recent meta-analysis (Pammi and Abrams 2015). However, before an intervention becomes a standard of care it needs to be confirmed in multiple studies and in different populations. Three large trials are currently ongoing: the NEOLACTO trial (Lactoferrin for prevention of sepsis in infants) in Peru, the ELFIN trial (Enteral Lactoferrin In Neonates) in the UK, and the LIFT trial (Lactoferrin Infant Feeding Trial to prevent sepsis and death in preterm infants) in Australia. Completion of ongoing trials will provide evidence from more than 6000 preterm neonates and may enhance the quality of the current evidence.

Lactoferrin in brain development and injury

High levels of Lf are found in human milk (Ronayne de Ferrer et al. 2000) and its transport from blood to cerebral and peripheral tissue is highly regulated after oral and i.v administration through binding sites on brain endothelial cells (Huang et al. 2007; Kamemori et al. 2008; Talukder and Harada 2006). As an iron binding protein Lf regulates the absorption of dietary iron, a metal crucial for adequate body and brain growth and development (Collard 2009). Lf is also synthesised by activated microglia and is believed to represent a defence mechanism in neurodegenerative diseases (An et al. 2009; Fillebeen et al. 2001; Kawamata et al. 1993; Wang et al. 2010). It has further been shown to decrease inflammation in disease (Haversen et al. 2002; Hayashida et al. 2004; Talukder and Harada 2007). For dopamine neurons Lf is protective in a model of mitochondrial dysfunction and oxidative stress, two mechanisms relevant for Parkinson disease (Rousseau et al. 2013) but also in developmental brain injury (Rees et al. 2011). Therefore Lf is likely to have supportive effects during brain development and after perinatal brain injury (figure 2).

Brain development and behaviour

Recent studies in normal rat pups showed that oral Lf supplementation (750 mg/kg/d) during postnatal period (16–34 day of life) could improve subsequent cognitive performance during stress (Shumake et al. 2014). Lf did not have effects on motor activity but rat pups receiving oral Lf showed reduced exploration of the risky environment, a greater preference to familiar odour food and a faster response to stress escape footshock test. No effect on spatial memory was observed. The same authors also evaluated effect of dose by giving 500, 1000, 2000 mg/Kg/day but did not show any difference between doses in regards to escape from footshock stress test. Only passive-avoidance was improved at both higher doses in males, but not in females. These initial data show an effect of postnatal Lf on brain function and response to stress.

In addition, in piglets receiving Lf enriched diet (155 mg/Kg/day) from day 3 to 38 postnatally early neurodevelopment and cognition was tested and showed improved spatial, association learning and memory in the supplemented animals compared to controls (Chen et al. 2014). Visual clues in Lf group were acquired more rapidly in both easy and difficult learning tasks. Importantly this study also looked at genomic changes induced by Lf and showed several effects on genes implicated in brain development and cognition. One major finding is the upregulation in the hippocampus of several genes implicated in the Brain

Derived Neurotrophic Factor (BDNF) signalling pathway important for neuroplasticity, cell migration and differentiation of progenitor as well as growth and targeting of axons and dendrites. Several genes implicated in molecular and cellular functions in the central nervous system were also upregulated (Table 1). In another study were piglets received a mixture of prebiotic, Lf and milk fat globule membrane supplementation, cerebral development assessed with neuroimaging was enhanced with brain volume differences in grey and white matter suggesting that supplemented piglets experienced axonal pruning earlier than non-supplemented fed piglets. Moreover, diffusion tensor measures for cerebral microstructure evaluation, suggested enhanced maturation of the internal capsule, further supporting the hypothesis of increased maturation in supplemented piglets compared with unsupplemented piglets. Behavioural assessment did not indicate differences in learning, but supplemented diet may have reduced impulsivity and/or anxiety (Mudd et al. 2016).

Recently, Lf (iron saturated or not) showed neuronal differentiating actions with upregulation of neuronal differentiation factors (β tubulin III, NF 68-160-200, NSE). This neuronal differentiation effect was through uptake by specific LRP1 and LRP2 cellular receptor and the PI3k and ERK signaling pathway in vitro (Sriramoju et al. 2015). This indicates that Lf support neuronal development.

As the investigations of the effects of oral Lf on brain development is relatively new the best effective dose range is not known and will require refinements. Despite a limited oral bioavailability, Lf is quickly transferred from the intestine to the brain after oral (Fischer, 2007) or intravenous administration (Kamemori, 2008). Lf binding sites are present in brain endothelial capillary cells and it is actively transported into the CSF in an intact form (Ji, 2006; Kamemori, 2008; Talukder, 2003; Fillebeen, 1999). From the studies mentioned earlier, high doses do not seem to provide more effects on brain function probably through saturation of transporters and limited availability.

Developmental brain injury

Neuroprotection with Lactoferrin in a model of preterm hypoxic-ischemic (HI) brain injury—Lactoferrin as a nutritional supplement with anti-inflammatory, anti-oxidant and antiapoptotic activities given to the dam from birth and throughout the lactation period showed neuroprotective effect following HI injury in 3 days old rat (P3) (van de Looij et al. 2014). Using Advanced Magnetic Resonance Imaging (MRI) analysis, the percentage of injured cortex after HI at P3 as well as the percentage of cortical loss at day 25 (P25) was significantly reduced in the Lf supplemented group, indicating acute and long-term protection effects. At the weaning period, P25, the cortical metabolism was almost normalized and the altered white matter induced by the HI injury showed recovery in the Lf supplemented pups with normalisation of the WM water diffusion parameters representing tissue microstructure evaluated with diffusion magnetic resonance imaging. Further, IL6, TNF α , brains markers of inflammatory response to HI are reduced as well as markers of cell death Caspase 3 and Fractin in conjunction with increased AkT activation suggesting anti-inflammatory and anti-apoptotic effects of Lf in P3 rat HI brain injury. Therefore, Lf as a nutrient received through lactation, shows long-term neuroprotective effect following HI in

the P3 pup rat. This initial result could be of high interest in the clinical field of neonatal brain neuroprotection.

Neuroprotection with Lactoferrin in a model of preterm brain inflammatory injury—Inflammation is a major cause of developmental brain injury (Dammann, 2014; Hagberg, 2015) and neurodevelopmental delay (Adams-Chapman, 2006) in the preterm infants. It affects myelination through disruption of oligodendrocytes maturation that takes places during prematurity period (Volpe, 2011; van Tilborg, 2015) but also neuronal migration and cortical development (Strunk, 2014; Leviton, 2007). To mimic infection/inflammatory developmental injury, bacterial Lipopolysaccharide (LPS) challenge is widely use (Edwards, 2006; Dean, 2011; Hagberg, 2002). We have induced brain injury in the P3 rat pup with intracerebral LPS injection and showed that the typical injury is reduced by dam Lf supplementation from birth and during lactation (Ginet et al. 2016): at P25 ventricular dilatation that is seen on MRI and histopathology after this inflammatory injury (Lodygensky et al. 2014) was reduced in the injured pups receiving LF through lactation. At weaning (P25) central white matter fibres of the striatum are altered with increased size of the diameter of the axons, that appears to be partially reversed by Lf supplementation. On electronic microscopy, the axonal diameter is increased in LPS animals with irregular and decreased thickness of the myelin sheets, indicating disruption of the myelination process and axonal microstructure. In the Lf supplemented animals axonal diameter does not show recovery, but myelin sheets around the axons are significantly more organised and compacted around the axons. Myelinating oligodendrocytes in the control group expressed signs of highly active cells whereas oligodendrocytes in the LPS group had reduced synthesis markers of activity (reduced RER and decreased number of polyribosomes in an electron-dense cytoplasm and increased content of heterochromatin in the nuclei). In the presence of Lf supplement the oligodendrocytes markers of activity appeared restored and comparable to control pups leading to the better myelination seen. Lf also appears to reduce activated inflammatory Iba1 positive microglia cells after LPS injury.

Advanced diffusion tensor imaging (DTI) (van de Looij et al. 2015) revealed a modification of brain microstructure characterized by tissue water diffusion parameters changes in the central white matter (striatum) 20 days after LPS injection. Further, in white matter structures, corpus callosum and external capsule, LPS cerebral exposure led to pattern in DTI derived parameters that is typical of myelin defect already observed in this model (Lodygensky et al. 2010) and other myelination deficits (van de Looij et al. 2015) that correlated with axonal and myelin changes seen with electronic microscopy. Lf supplementation during lactation corrected LPS-induced microstructural DTI derived parameters alterations in the white matter.

On the metabolic level, using proton magnetic spectroscopy (^1H -MRS) at ultra-high magnetic field, intracerebral LPS injection in P3 rat induced acute (24h post-injection) specific changes in the “neurochemical profile” of central white matter (corpus callosum) (Lodygensky et al. 2014). LPS-induced alterations (increase or decrease) in brain metabolites concentration also occurred in other central white matter fibres (striatum) after LPS injection and Lf supplementation reduced these acute changes. Markers of tissue injury, Excitatory neurotransmitter and anaerobic metabolism were reduced by Lf after LPS

challenge. Antioxidative compound were increased in the LPS+Lf brains as well as inhibitory neurotransmitter and energy metabolism. These cerebral metabolic changes indicate that injury mechanism are reduced and protective mechanisms enhanced by lactoferrin.

In summary Lf during lactation shows neuroprotection with reversal of LPS inflammatory induced ventricular dilatation, of myelination deficit and axonal microstructure, with reduced microglia response and restoration of brain metabolic status.

Neuroprotection with Lactoferrin in dexamethasone induced intrauterine growth restricted (IUGR) rats—Exposure *in utero* to exogenous glucocorticoids mimics both maternal stress and the clinical situation when glucocorticoids are administered to pregnant women at risk of premature delivery. Rat and ovine models of glucocorticoid exposure during gestation have been shown to cause IUGR (LaBorde et al. 1992) and brain weight, hypomyelination (Dunlop et al. 1997), and a delay in the differentiation of brain tissues (Basilious et al. 2015; Van den Hove et al. 2006b). Using rat IUGR/stress model of dexamethasone (DEX) exposure during late gestation, hippocampal metabolism and gene expression was investigated (Somm et al. 2014). Attention of the study was on hippocampus at P7 (a significant time point corresponding to a late neuronal proliferative phase) since this brain limbic region involved in the long term memory process contains high levels of glucocorticoid receptors, making it particularly vulnerable to DEX-induced foetal programming (Noorlander et al. 2008; Uno et al. 1994). Nutritional supplementation with Lf to the dam during gestation and lactation reversed the effects of maternal exposure to dexamethasone (Somm et al. 2014). At P7, both levels of glutamate and NAA ($^1\text{H-MRS}$), microstructure with neuronal density recovery in the hippocampus and gene expression of BDNF and MT1 were altered in DEX animals, but normalized by Lf supplementation during gestation and lactation. Microarrays allowed deeper characterisation of the hippocampal transcriptomic hallmark in DEX-IUGR pups as well as the impact of maternal Lf. Some transcripts were specifically upregulated by maternal Lf exclusively in DEX-IUGR pups, such as Nrep (neuronal regeneration related protein) and S100b a marker of the nervous system damage at adulthood with neurotrophic function in the developing CNS and shown as decreased following prenatal stress (Van den Hove et al. 2006a).

These initial preclinical studies on effects of Lf on brain development and preterm brain injury are very promising for future use of Lf in preterm infants.

Lactoferrin and preterm delivery

Lactoferrin has some potential to prevent infection and help for recovery of brain damage in preterm infants but few preclinical and clinical studies appear to show that by reducing inflammation in pregnant animal/women it might also be prevent preterm birth as infection/inflammation are a well-known risks for preterm delivery and brain injury (Edwards and Tan 2006; Hagberg et al. 2005).

In a rabbit model of intrauterine infection with *E. coli* (Hasegawa et al. 2005), recombinant human Lf as local treatment increased survival rate of foetus and extended length of

pregnancy compared to infected controls. Lf treated animals showed no inflammatory exudates or necrosis in the endometrium, decidua, or placenta that were present in the infected only animals. These also had significantly higher levels of TNF α compared to the animals receiving Lf. In a similar manner intraperitoneal injection of Lf after LPS challenge in pregnant mice prolonged the duration of gestation by reducing IL6 plasma levels in the treated group. Interestingly human recombinant Lf had greater effects compared to bovine Lf (Mitsuhashi et al. 2000; Sasaki et al. 2004). In mice, markers of LPS induced endometriosis such as histological inflammatory changes, myeloperoxidase activity, NF κ B, TNF α and IL1 β were reduced in Lf treated animals (Otsuki et al. 2005) and in a dose dependent manner (Li et al. 2015).

In pregnant women, administration of oral and intravaginal Lf to the women with risk of preterm delivery decreased IL-6 in both serum and cervicovaginal fluids, cervicovaginal prostaglandin F2a, and suppressed uterine contractility. Lf administration blocked further shortening of cervical length and the increase of fetal fibronectin thus prolonging the length of pregnancy (Paesano et al. 2012). Similarly, on a selection of women at risk of preterm delivery based on borderline cervical length and elevated cervico-vaginal IL6 that received vaginal tablets of Lf (300mg/day), IL6 levels were reduced whereas cervical length increased compared to the non-treated women. Further a greater number of women in the control group had regular uterine contraction and reduced cervical consistency before 37 weeks of pregnancy (Locci et al. 2013). These initial data are supportive for reduced inflammation induced by Lf that could prevent preterm delivery.

Conclusion

Infection and NEC are major burden for immediate and long terms outcome in preterm infants that often require heavy antimicrobial treatments, surgery and prolonged hospital stay. They also contribute to brain injury and altered development through activation of inflammatory processes that are also major determinants of neurodevelopmental disabilities later in life. To date there is no protective strategies in the preterm infant to reduce brain injury and dysfunction despite increased survival at low gestational age. In addition to the effects of Lf on preterm infection, NEC and on brain injury in preclinical studies, Lf might have a potential therapeutic effect in pulmonary bronchodysplasia and retinopathy of prematurity of the preterm that are also mediated by oxidative stress and inflammation. Lactoferrin research in these fields is needed and could be also of interest to reduce morbidity of the preterm infants.

Lactoferrin through its multiple properties and actions appears to have some potential to reduce several major morbidities in preterm infants. Importantly no secondary effects to date have been reported in preclinical and clinical studies. It is now clear that from the clinical studies on preterm infection and NEC and the preclinical data that the effects of Lf need to be further investigated and established in this vulnerable population.

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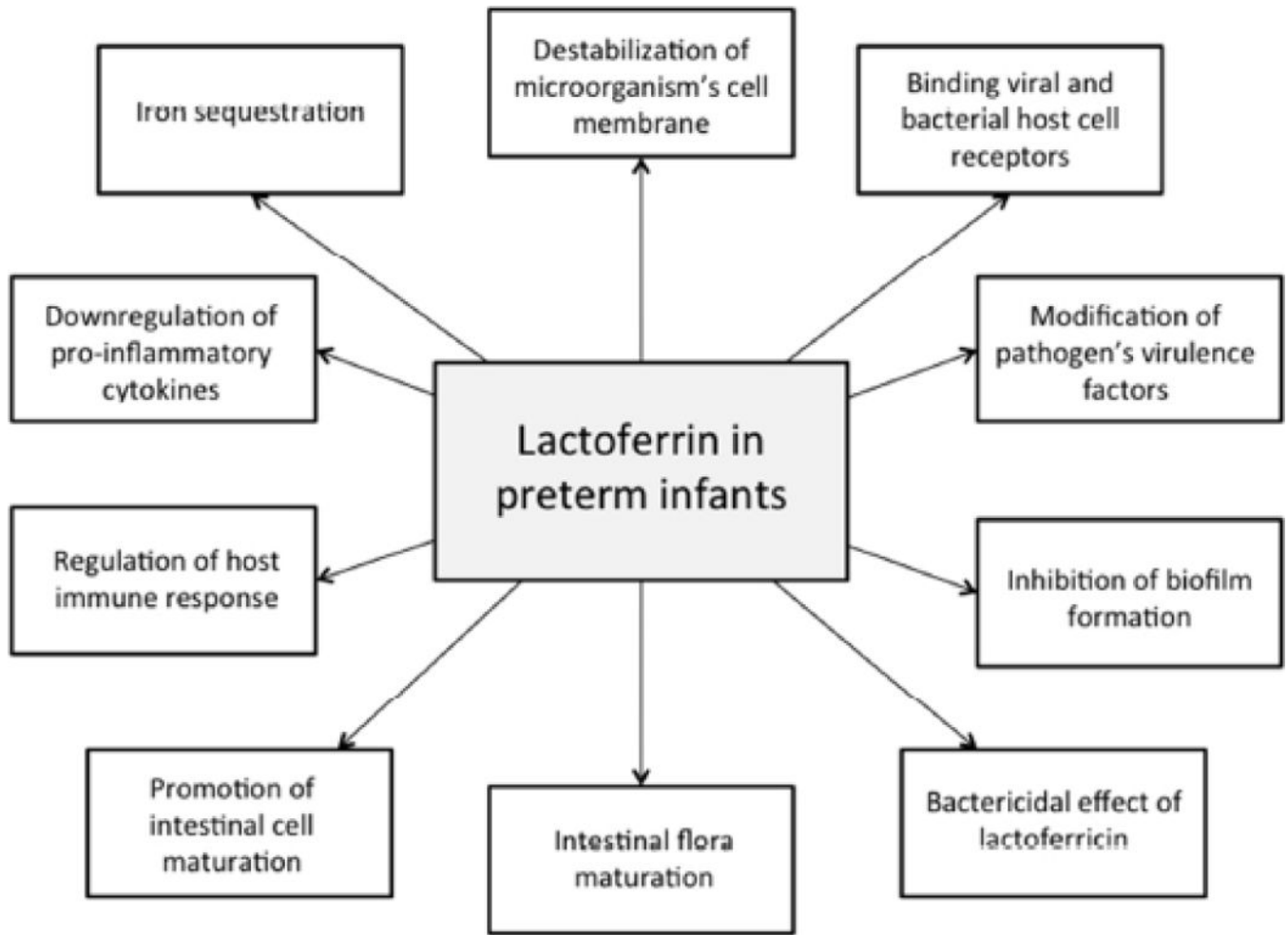


Figure 1. Potential protective mechanisms of lactoferrin in preterm infants against infection and NEC

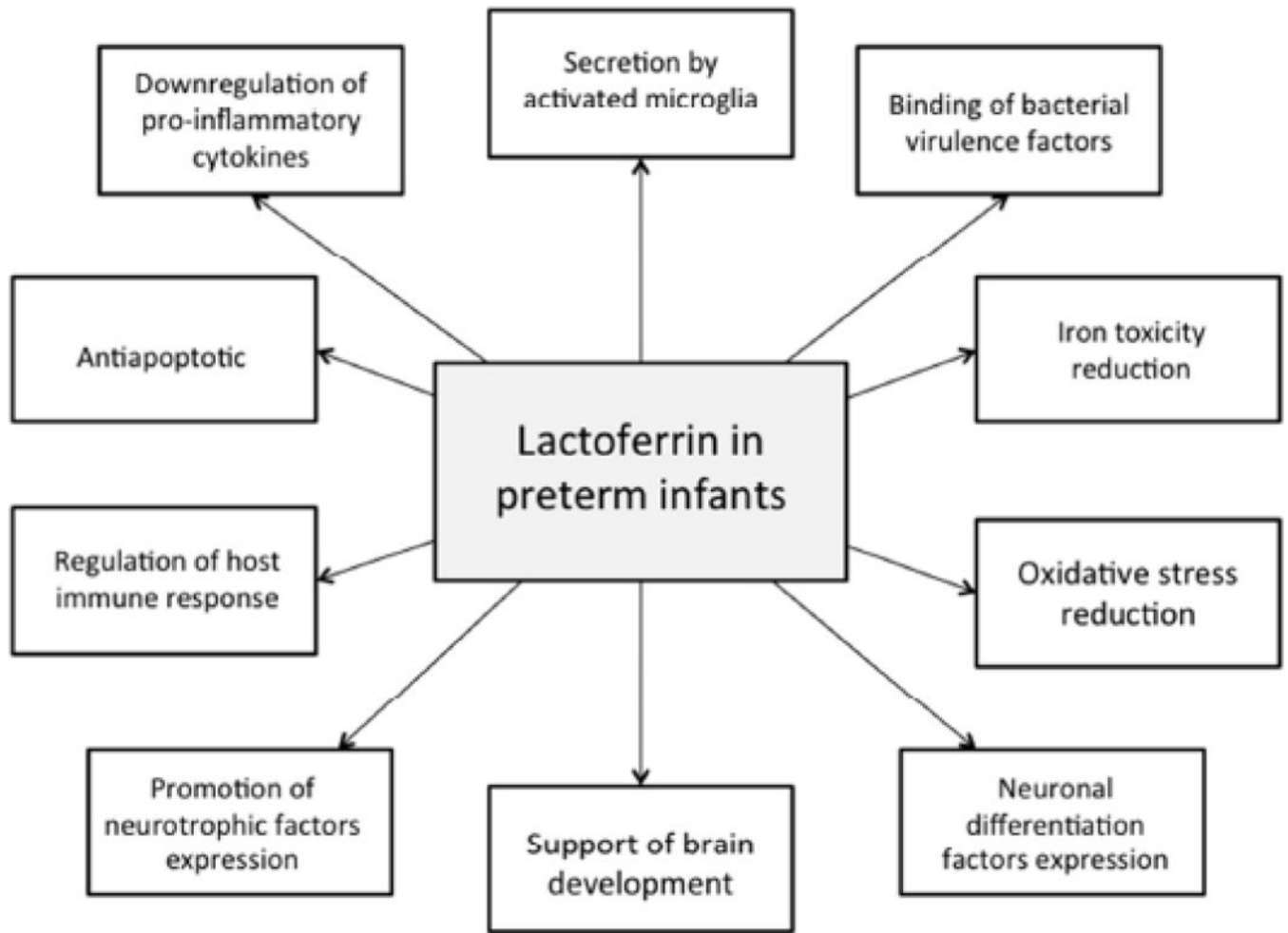


Figure 2. Potential protective mechanisms of lactoferrin in preterm developmental brain injury

Table 1

Summary of molecular and cellular functions in the central nervous system affected by postnatal dietary lactoferrin in piglets. Adapted from (Chen et al. 2014)

Molecular and cellular functions in the CNS	Activation state	Number of molecules involved*
Formation of cellular protrusion	Increased	39
Microtubule dynamics	Increased	49
Cytoplasm organisation	Increased	57
Formation of cytoplasm membrane projections	Increased	28
Organisation of cytoskeleton	Increased	51
Outgrowth of neurites	Increased	16
Formation of neurites	Increased	9
Anxiety	Decreased	10

Based on gene expression measured by microarray and subjected to pathway analyses using the Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com)