Clinical applications of bioactive milk components

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Milk represents a unique resource for translational medicine: It contains a rich pool of biologically active molecules with demonstrated clinical benefits. The ongoing characterization of the mechanistic process through which milk components promote development and immunity has revealed numerous milk-derived compounds with potential applications as clinical therapies in infectious and inflammatory disease, cancer, and other conditions. Lactoferrin is an effective antimicrobial and antiviral agent in high-risk patient populations and a potentially potent adjuvant to chemotherapy in lung cancer. Enteric nutrition formulas supplemented with transforming growth factor β , a milk cytokine, have been shown to promote remission in pediatric Crohn's disease. A number of milk glycans, including human milk oligosaccharides, show promise in preclinical studies as antimicrobial and anti-inflammatory agents. While active preclinical investigations of human milk may soon result in large-scale production of human milk molecules, bovine milk components in many instances represent a practical source of bioactive milk compounds for use in clinical trials. This review summarizes current efforts to translate the compounds derived from human and bovine milk into effective clinical therapies. These efforts suggest a common pathway for the translation of milk-derived compounds into clinical applications.

INTRODUCTION

Milk is simultaneously a comprehensive source of nutrition and a potent adjuvant to defense against infectious disease in infants. Indeed, the clinical benefits of breastfeeding in both the neonatal period^{1,2} and after weaning^{3,4} are well documented, and breastfeeding has been widely advocated by pediatricians for decades. The gastrointestinal (GI) tract is both functionally and immunologically immature at birth. Maturation requires colonization by microorganisms and the coalescence of a functional microbial community that is fully integrated with the nutritional and immunological requirements of the human host.^{5,6} The immature intestinal mucosa is prone to damaging inflammation.⁷ However, breastfed infants are at a substantially reduced risk of

developing inflammatory diseases such as necrotizing enterocolitis.⁸ The comparison of inflammatory disease incidence in breastfed and formula-fed infants³ suggests that, besides having anti-infective and prebiotic functions, milk components may enhance immune development and the maturation and function of the GI tract. Thus, a rich matrix of milk proteins and carbohydrates provides optimal nutrition⁴ and robust immune protection for the infant.⁹

Characterization of the mechanistic process through which milk components promote development and immunity has revealed numerous milk-derived compounds with translational potential for the development of novel clinical therapies. Although emphasis has been placed on the application of milk components to treat GI disease, it is possible that milk components

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may be useful in treating widely prevalent diseases outside the digestive tract, such as cancer, cognitive decline, and hypertension. The evaluation of specific human milk components in the clinical setting has been limited by the lack of techniques for producing such components. Alternate production pathways include large-scale isolation of milk compounds from dairy products in cases in which bovine structures exhibit equivalent function to human milk compounds, or the generation of transgenic livestock capable of expressing human milk constituents during lactation. Large-scale production of highly refined bovine milk components has enabled evaluation of antimicrobial, anti-inflammatory, and antihypertensive properties of conserved milk components in adequately powered clinical trials. Due to their low cost and wide availability, these products have the potential to affect human health significantly.

MILK IMMUNOGLOBULINS

Secretory antibodies, or secretory type A immunoglobulins, were among the first molecules with antibiotic properties identified in both human and bovine milk.¹⁰ Colostrum may contain concentrations of maternal antibodies as high as 12 g/L in humans, and mature milk retains the ability to provide robust protection against pathogens throughout infancy at 1 g of soluble immunoglobulin per liter.¹⁰ Antibodies to pathogen-specific antigens are generated through the interaction of the maternal immune cells with enteric pathogens. The mature intestinal mucosa and submucosa support a plethora of specialized immune cells that make up the gut-associated lymphoid tissue. Microfold cells, specialized mucosal epithelial cells, facilitate not only the transport of dietary, microbial, and self antigens across the epithelial barrier but also the presentation of these antigens at the basal epithelium to the underlying lymphoid cells.¹¹ Dendritic cells, antigen-presenting cells of the lymphoid lineage that populate the lamina propria, sample the remaining surface of the intestinal mucosa intermittently via cytoplasmic extensions that protrude across the epithelial barrier. Exposure to cognate antigen results in the proliferation of T cells and B cells expressing specific complementary receptors,¹¹ some of which circulate systemically and enhance the secretion of protease-resistant secretory immunoglobulin A by the plasma cells of the mammary epithelium in lactating mammals (reviewed by Newburg and Walker⁹).

Maternal antibodies, thus, transfer the cumulative adaptive secretory immunity of a lifetime of enteric antigen exposure to the immune-naïve digestive tract of the neonate and exert direct microbicidal and antiadhesive activity on enteric pathogens. Compared with standard formula, infant formula supplemented with pooled

bovine colostrum antibodies was associated with a significant reduction in diarrhea and related morbidities among a large population of Iraqi children.¹² A preparation of colostrum antibodies isolated from cows immunized against a broad panel of enterotoxigenic *Escherichia coli* antigens reduced the incidence of traveler's diarrhea by as much as 90% among a healthy adult cohort,¹³ demonstrating that bovine colostrum is an adaptable source of specific antimicrobial prophylaxis. This preparation is currently available in Australia as a nonprescription prophylactic treatment for the prevention of traveler's diarrhea (Travelan, Anadis, Campbellfield, Victoria, Australia). Bovine antibodies have also been applied therapeutically, resulting in a significant reduction in diarrhea in rotavirus-infected children treated with antibodies isolated from colostrum produced by cows immunized against several strains of human rotavirus.¹⁴ The evaluation of bovine colostrum antibodies against *Cryptosporidium parvum*,¹⁵ *Clostridium difficile*,¹⁶ and human immunodeficiency virus (HIV)¹⁷ in vitro and in animal models of disease has potentially significant implications for the treatment of life-threatening human diseases through the transfer of passive immunity from immunized cows (Table 1).^{12,13,18–28}

ANTIMICROBIAL AND ANTITUMOR PROPERTIES OF LACTOFERRIN

Antimicrobial properties of lactoferrin

In addition to a rich complement of antibodies highly specific for certain antigens, milk contains antimicrobial proteins with multifaceted, broad-spectrum activity.²⁹ Among these, lactoferrin is an 80-kDa cationic glycoprotein belonging to a family of nonheme iron-binding proteins. The binding of iron at high affinity exerts microbiostatic pressure on iron-sensitive microorganisms of the digestive tract and is key to the physiologic functions of lactoferrin. Direct microbicidal activity of lactoferrin has also been proposed.³⁰ In vitro experiments have demonstrated growth inhibition of several human pathogens in the presence of recombinant human lactoferrin, including *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Bacillus subtilis*, *Vibrio cholerae*, and *Candida albicans*.³⁰ Lactoferrin has been demonstrated to inhibit in vitro replication of human cytomegalovirus, HIV, herpesvirus, hepatitis B and C, hantavirus, human papillomavirus, rotavirus, adenovirus, and influenza A.³¹

Numerous clinical applications of various forms of lactoferrin have been devised and evaluated (Table 1). These studies have been facilitated by large-scale

Table 1 Summary of clinical trials conducted using milk components in antimicrobial applications

Milk component	Organism	Result of clinical trial	Reference
SlgA	Not specified	Reduced incidence of diarrhea	Tawfeek et al. (2003) ¹²
SlgA	Enterotoxigenic <i>Escherichia coli</i>	Reduced incidence of infection by 90%	Otto et al. (2011) ¹³
Lactoferrin	Not specified	Reduced duration and severity of diarrhea	Ochoa et al. (2012) ¹⁸ , Laffan et al. (2011) ¹⁹
	Not specified	Reduced incidence of respiratory infection	King et al. (2007) ²⁰
	Not specified	Reduced incidence of bacterial and fungal sepsis in premature infants	Manzoni et al. (2009) ²¹ , Manzoni et al. (2012) ²²
	Not specified	Reduced mortality in patients with acute bacteremia by 46%	Guntupalli et al. (2013) ²³
	<i>Helicobacter pylori</i>	Improved infection resolution rate when added to standard therapy	De Beortoli et al. (2007) ²⁴
	Hepatitis C virus	Improved treatment response when used in combination with standard therapy	Kaito et al. (2007) ²⁵
	Rhinovirus	Reduced symptom severity and duration	Vitetta et al. (2013) ²⁶
Lactoferrin and lysozyme	Not specified	Reduced incidence of diarrhea	Zavaleta et al. (2007) ²⁷
3'-sialyllactose	<i>Helicobacter pylori</i>	No benefit	Parente et al. (2003) ²⁸

Abbreviation: slgA, soluble immunoglobulin A.

production of lactoferrin, most prominently the purification of bovine lactoferrin from whey.³² Recombinant human lactoferrin has been successfully expressed in a variety of bacterial, fungal, plant, and animal vectors and is commercially produced on an industrial scale in *Aspergillus awamori* (Agennix, Houston, Texas, USA), rice (Ventria Bioscience, Sacramento, California, USA), and transgenic cows (Pharming, Leiden, The Netherlands).

Animal studies have demonstrated a reduction in gut-derived sepsis following oral bovine lactoferrin treatment of neonatal rats infected with *E. coli*, indicating that milk lactoferrin protects against infection in nursing mammals.³³ Prophylactic administration of recombinant human lactoferrin to a large population of children in a randomized, double-blind, placebo-controlled study reduced both the duration and severity of diarrhea over 6 months.¹⁸ Compared with standard formula, infant formula supplemented with recombinant human lactoferrin also reduced the risk of respiratory infection during the first 12 months of life.²⁰ Nosocomial sepsis is a leading cause of morbidity and mortality among the neonatal intensive care population. Administration of bovine lactoferrin to very-low-birthweight premature infants reduced the incidence of bacterial sepsis in the first 45 days of life by 70% relative to placebo treatment in a large, randomized study.²¹ The incidence of fungal sepsis was also reduced by bovine lactoferrin supplementation in this high-risk population.²²

Similar clinical benefits may apply in adult populations. Treatment of acute bacteremia in adult intensive care patients using recombinant human lactoferrin reduced all-cause mortality by 46% relative to placebo

treatment in a multicenter trial.²³ The efficacy of recombinant lactoferrin supplementation during standard eradication therapy for *H. pylori* infection has been evaluated in a large, multicenter, prospective trial, resulting in a statistically significant enhancement in treatment effectiveness among patients receiving the lactoferrin supplement.²⁴ Elderly patients are at high risk of developing postantibiotic diarrhea. Recombinant lactoferrin therapy significantly reduced the incidence of diarrhea over a period of 8 weeks in a randomized, placebo-controlled, double-blind study in this patient population.¹⁹

The oral administration of a combination of recombinant lactoferrin and lysozyme significantly decreased the duration and severity of acute diarrhea in a double-blind trial involving 143 children.²⁷ This novel observation suggests the synergistic potential of bioactive milk compounds. Recombinant human lactoferrin is an effective adjunct therapy in chronic hepatitis C (HCV) infection and other viral infections. The addition of recombinant lactoferrin to standard therapy of interferon (IFN) and ribavirin resulted in a sustained reduction of HCV titer when compared with standard therapy alone.²⁵ The mechanism of action of lactoferrin in the treatment of HCV infection apparently involves direct disruption of HCV envelope proteins by structural domains that are independent of antibacterial function, reflecting specific antiviral adaptation.³⁴ In a recent study, 90 patients who indicated frequent cold symptoms were given human recombinant lactoferrin 600 mg/day or placebo for 90 days. Patients in the lactoferrin treatment group reported a significant reduction in symptom severity and reduced duration of symptoms relative to the control group.²⁶ Thus, lactoferrin is a

multifaceted antimicrobial agent with demonstrated clinical efficacy in the treatment of infectious disease in humans.

Lactoferrin-mediated inhibition of tumor growth

After exhibiting immunomodulatory, anti-angiogenic, and proapoptotic activities *in vitro*, lactoferrin was evaluated as a therapeutic agent for the treatment of human cancer.³⁵ Secreted lactoferrin is a potent anti-inflammatory agent, capable of modulating the interaction between inflammatory stimuli and cognate cell surface receptors.³⁶ As a result, lactoferrin plays an important role in the microenvironment by regulating cellular growth and differentiation and influencing the immune response.³⁵ Oral consumption of bovine lactoferrin 3 g/day significantly impaired the growth of adenomatous polyps of the colon in an adult cohort undergoing regular monitoring by colonoscopy.³⁷ In a randomized, double-blind, placebo-controlled study, administration of recombinant lactoferrin extended survival by an average of 65% in patients with advanced stage non-small cell lung carcinoma.³⁸ The same preparation was associated with marked improvements in overall survival when applied as an adjunct to standard chemotherapy in patients with newly diagnosed lung cancer.³⁹ Additional *in vivo* data suggests that lactoferrin may enhance the effectiveness of chemotherapeutic treatment of breast cancer.⁴⁰ The inhibition of tumor growth in animal studies has been attributed to the anti-angiogenic and anti-inflammatory functions of lactoferrin.⁴¹ Clearly, further study is warranted to explore the application of lactoferrin in the treatment of lung cancer and other malignancies.

MILK POLYSACCHARIDES

Milk contains ample polysaccharides, including oligosaccharides and glycosaminoglycans. Indigestible oligosaccharides are the third most abundant milk component, present at concentrations as high as 20 g/L in colostrum or 5–10 g/L in mature human milk.⁴² Human milk oligosaccharides are defined by a structure composed of lactose on the reducing end, a polylactosamine core, and often fucose (neutral oligosaccharides) or sialic acid (acidic oligosaccharides) at the nonreducing terminus.⁹ More than 200 unique human milk oligosaccharide structures have been identified.⁴³ Human milk oligosaccharides are highly resistant to degradation in the upper digestive tract and do not appear to serve any direct nutritional function.⁴⁴ Viral, bacterial, and protozoan pathogens of the digestive tract are dependent upon the expression of specific cell surface structures, including both glycans and lectins,

to achieve targeted adherence and invasion of host epithelium.

Inhibition of bacterial adhesion by human milk oligosaccharides

The vast pool of structural variations in human milk oligosaccharides includes numerous small carbohydrate structures capable of acting as soluble decoys for the cell surface antigens targeted by pathogenic organisms. These carbohydrate structures inhibit the adhesion of pathogens to the mucosal surface through multifaceted high-avidity interactions.⁹ This principle is illustrated by the inhibition of *Campylobacter jejuni* infection specifically by fucosylated human milk oligosaccharides.⁴⁵ A subsequent study of a large human cohort demonstrated that overall incidence of *Campylobacter* diarrhea was dramatically reduced among the infants of nursing mothers who expressed high levels of 2'-fucosyllactose.⁴⁶ Together, the data suggest that 2'-fucosyllactose is an inhibitor of *Campylobacter* infection with potential for translation as a prophylactic anti-infective agent. Human milk oligosaccharide-mediated inhibition of numerous pathogenic bacteria has been demonstrated. Human milk oligosaccharides inhibit adhesion of enteropathogenic *E. coli* to cultured epithelium independently of milk protein fractions⁴⁷ and significantly reduce the cytotoxicity associated with *E. coli* enterotoxin in animal models⁴⁸ and in cultured human epithelium⁴⁹ through inhibition of enterotoxin binding. Similarly, sialylated oligosaccharides inhibit cholera toxin-induced diarrhea in rabbits.⁵⁰ Pooled human milk oligosaccharides also inhibit adhesion of the diarrheal pathogens *Vibrio cholerae*, *Salmonella fyris*, *E. coli*, and *Listeria monocytogenes* to cultured human epithelium.⁵¹ Human milk oligosaccharides are also effective inhibitors of viral pathogens, including HIV.⁵² Tail-vein injection of Gal β 1-4Gal β 1-4Glc, a human milk oligosaccharide analog, resulted in a 10-fold reduction in established urinary tract infection in mice, demonstrating effective dissemination of a human milk oligosaccharide through the circulation.⁵³

Clinical trials of human milk oligosaccharides have been limited by the scarcity of defined human milk oligosaccharide products. Human milk oligosaccharides containing terminal sialic acid inhibit adhesion of *H. pylori* to cultured gastric epithelium.⁵⁴ Experiments in rhesus monkeys suggested that a specific sialic acid-containing human milk oligosaccharide, 3'-sialyllactose, could effectively resolve *H. pylori* infection⁵⁵; however, a small clinical trial failed to demonstrate a significant benefit of the consumption of 3'-sialyllactose by patients with established *H. pylori* infection.²⁸ Thus, despite tremendous promise as anti-infective agents, clinical

evidence for the application of human milk oligosaccharides as antimicrobial agents is lacking.

Prebiotic oligosaccharides

Prebiotics are defined as indigestible food components that benefit the consumer through the selective stimulation of beneficial growth or activity of specific endogenous microbiota.⁵⁶ Dietary factors may shape the composition and function of the human intestinal microbiota,^{57,58} although the significance of long-term diet-induced changes in the gut microbiome relative to other sources of interindividual variation is unclear.⁵⁹ Breastfeeding may have implications for long-term health and development by influencing the risk of inflammatory disease^{3,60} and obesity.^{4,48,61} Attempts to influence the pattern of microbial colonization during infancy using prebiotics have focused on recapitulating microbial communities associated with breastfeeding, although in many cases the mechanistic basis of benefits associated with particular taxa remains undefined. Bifidobacteria and Bacteroidetes dominate the fecal microbiota in breastfed infants,^{62,63} and colonization with greater relative abundance of Bifidobacteria is associated with positive health outcomes, including reduced risk of autoimmune disease.⁶⁴

Several studies have demonstrated that infant formula to which a mixture of neutral short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides was added stimulated the growth of bifidobacteria and lactobacilli,⁶⁵ reduced overall infection incidence during infancy, and reduced the incidence of atopic dermatitis and allergic rhinitis in the first 5 years of life compared with standard formula.⁶⁶ However, other studies of the use of lactose and plant-based prebiotics as formula supplements have reported largely inconsistent clinical results.⁶⁷ A recent meta-analysis of the use of plant-based galacto- and fructo-oligosaccharide prebiotics in infants revealed no evidence of a reduction in the incidence of infectious or inflammatory disease, while noting that the majority of infants tolerate these prebiotic compounds very well.⁶⁸

Thus, while the use of prebiotics for disease prevention in infants shows promise, currently available prebiotic preparations cannot completely replicate the beneficial effects associated with breastfeeding. The genomes of *Bifidobacterium* species suggest specific selection to prebiotic human milk oligosaccharide utilization.⁶⁹ Human milk oligosaccharides serve as a selective substrate, providing a distinct colonization advantage to bacterial genera capable of metabolizing specific carbohydrate linkages.⁷⁰ Fermentation of human milk oligosaccharides by bifidobacteria results in

inhibition of other microbes present in the neonatal GI tract, including *E. coli* and *Clostridium perfringens*,⁷¹ as well as modulation of the epithelial barrier function.⁷² Additional bacterial genera associated with the healthy adult digestive tract are also able to effectively utilize human milk oligosaccharides, including *Lactobacillus* and *Bacteroides*.⁷³ In germ-free mice co-inoculated with gut symbionts *Bacteroides thetaiotaomicron* and *Bifidobacterium infantis* and fed a polysaccharide-deficient diet, dietary supplementation with the human milk oligosaccharide lacto-*N*-neotetraose resulted in rapid expansion of *Bifidobacterium* populations and relatively little growth in Bacteroidetes populations in the murine gut.⁷⁴

However, several emerging lines of evidence have suggested that supplementation with human milk oligosaccharides may promote growth of potential pathogens as well. High mortality and significant bacterial outgrowth in the distal bowel were observed in mice monocolonized with an enterotoxigenic strain of *C. perfringens* following supplementation with human milk oligosaccharides.⁷⁵ Additional in vitro data suggests that the supplementation of infant formula with human milk oligosaccharides enhanced growth of opportunistic pathogens *S. aureus* and *Staphylococcus epidermidis*, and culture-independent data correlates concentrations of human milk oligosaccharides with the presence of *Staphylococcus* species in human milk samples.⁷⁶

A small clinical trial of formula enriched with galacto-oligosaccharide or human milk oligosaccharide from donor milk administered to premature infants reported no appreciable difference in stool bacterial composition, noting that antibiotic use was widespread among the research subjects.⁷⁷ Limited supply currently precludes extensive trials of human milk oligosaccharides as prebiotic agents in humans and animals. Characterization of the beneficial organisms of the human microbiota and the predominant human enteric pathogens and their utilization of defined human milk oligosaccharide structures on a strain-specific basis is an essential step in the development of targeted human milk oligosaccharide-based prebiotic therapy.⁷⁰ Continued progress in the development of large-scale synthesis of complex carbohydrates⁷⁸ should facilitate human trials of defined human milk oligosaccharide preparations in the coming years. New means of producing and isolating human milk oligosaccharides, combined with increased understanding of the dynamic interrelationship between human milk oligosaccharide structure and the developing gut microbiota, will facilitate the design and testing of novel prebiotic agents based on human milk oligosaccharides in clinical settings in the near future.

Immunomodulatory properties of human milk oligosaccharides

Human milk oligosaccharides are multifunctional agents and demonstrate immunomodulatory effects that are potentially independent of the effect of altered microbiota composition on immunity. Approximately 1% of ingested human milk oligosaccharides (concentrations in milk may be as high as 20 g/L) enter the circulation,⁷⁹ where they may exert systemic effects on the immune system. Circulating human milk oligosaccharides have been demonstrated to have significant immunomodulatory effects. Intraperitoneal injection of the human milk oligosaccharide lacto-*N*-fucopentose III in mice promotes the proliferation of a subpopulation of peritoneal macrophages that suppress CD4⁺ T cells⁸⁰ while simultaneously initiating the activation of natural killer cells.⁸¹ A similar result was obtained following the injection of lacto-*N*-neotetraose, which resulted in the suppression of proliferation of naïve CD4⁺ T cells through secretion of interleukin (IL)-10 and IL-13 by macrophages.⁸²

Acidic human milk oligosaccharides have also been demonstrated to enhance IL-13 and IFN- γ expression in mononuclear cells derived from human umbilical cord blood, indicating that human milk oligosaccharides influence immune development in immature human lymphocytes.⁸³ Lectins have been proposed as potential cell surface receptors for milk glycans.⁸⁴ Sialylated human milk oligosaccharides inhibit lectin-mediated adhesion of leukocytes to endothelium, a critical step in the expansion of the inflammatory response through leukocyte recruitment from the bloodstream,⁸⁵ and suppress, *ex vivo* in whole blood, the formation of platelet-neutrophil complexes associated with inflammatory disease.⁸⁶ Through competition with lectin-mediated interactions in endothelium or other immune cells, human milk oligosaccharides may act to suppress leukocyte infiltration and the escalation of the immune response associated with necrotizing enterocolitis and other inflammatory diseases.⁸⁷ Gavage-fed animals that received formula containing human milk oligosaccharides exhibited improved survival and reduced intestinal pathology scores relative to animals receiving formula alone in a rodent model of necrotizing enterocolitis. This effect was associated with the presence of a specific sialylated human milk oligosaccharide, disialyllacto-*N*-tetraose, in the formula enriched with human milk oligosaccharides.⁸⁸ Future animal studies will facilitate the translation of human milk oligosaccharides to clinical applications in inflammatory disease.

Anti-inflammatory glycosaminoglycans

Glycosaminoglycans are defined as linear polysaccharides of repeating disaccharide units consisting of an

amino sugar and uronic acid or galactose, typically linked by covalent bonds to protein core structures to form proteoglycans.⁸⁹ Glycosaminoglycans, along with their associated proteoglycans, are the predominant component of the extracellular matrix. Numerous functions in wound repair, coagulation, and host defense have been attributed to glycosaminoglycans.⁹⁰ The first report to suggest that glycosaminoglycans were a significant, functional component of the human milk glycome demonstrated that chondroitin in human milk specifically inhibited the interaction of HIV with its cognate receptor, CD4.⁹¹ More recently, the glycosaminoglycan composition of human milk was determined by mass spectrometry, with the predominant species being chondroitin sulfate and heparin sulfate, along with relatively minor amounts of hyaluronan and dermatan sulfate.⁹² High-molecular-weight hyaluronan isolated from human milk has been demonstrated to enhance antimicrobial peptide expression in intestinal epithelium *in vivo* through a mechanism dependent on CD44 receptor and Toll-like receptor 4 and to enhance functional resistance of cultured epithelium to enteric infection.⁹³ Intraperitoneal injection of hyaluronan suppresses immune activation in a murine model of colitis⁹⁴ and may also promote the proliferation of anti-inflammatory IL-10-secreting T-regulatory cells.⁹⁵ Numerous clinical studies have demonstrated the effectiveness of glycosaminoglycan preparations in enhancing wound healing,⁹⁶ and glycosaminoglycans may function similarly to enhance GI wound healing.⁹⁷ Characterization of human milk glycosaminoglycans and proteoglycans may guide the development of specific glycosaminoglycan- and proteoglycan-based immunomodulators.

POTENTIAL MILK-DERIVED MEDIATORS OF GASTROINTESTINAL BARRIER INTEGRITY

Compared with formula, colostrum is associated with significantly decreased intestinal permeability in the healthy newborn.⁶⁷ Bovine colostrum has been used to treat deficiencies in GI barrier function in both human and animal disease, although the specific colostrum constituents mediating intestinal barrier protection remain poorly defined. Limited bacterial translocation across the intestinal barrier is an important component of adaptive immune maintenance but has also been identified as a significant etiologic factor in many cases of sepsis.⁹⁸

Colostrum is highly effective in neutralizing plasma endotoxins as well as reducing lymphatic bacterial infiltration in rats following chemically induced endotoxemia.⁹⁹ Rats treated with orally administered bovine colostrum following ischemic intestinal injury showed

decreased mucosal damage, limited epithelial permeability, suppression of the systemic cytokine response, and reduced bacterial accumulation in the lymphatic system compared with rats treated with saline alone.¹⁰⁰ Oral administration of bovine colostrum reduced gastric and small intestinal injury¹⁰¹ and limited bacterial translocation across the intestinal epithelium¹⁰² in rodent models of nonsteroidal anti-inflammatory drug-induced GI injury. Bovine colostrum conferred significant protection in a porcine model of necrotizing enterocolitis when compared with supplemented formula preparations.¹⁰³

A human clinical trial evaluated the prophylactic consumption of bovine colostrum in patients undergoing abdominal surgery, reporting that patients who received colostrum had significantly lower levels of serum endotoxin as a result of decreased translocation of gut-derived microbial endotoxin following surgery.¹⁰⁴ Studies to address the clinical efficacy of bovine colostrum in the prevention of gut-derived sepsis among high-risk patient groups are warranted. Further refinement of individual bioactive milk compounds will facilitate the development of pharmaceutical products designed specifically to treat GI barrier deficiency.

IMMUNOMODULATORY PROPERTIES OF TRANSFORMING GROWTH FACTOR β

Milk contains physiologically relevant concentrations of transforming growth factor β (TGF- β), a multifunctional secreted cytokine expressed by most cell types and whose broad activity includes immunomodulation and the regulation of cellular proliferation and differentiation.¹⁰⁵ Human milk contains TGF- β at concentrations as high as 1.5 $\mu\text{g/mL}$,¹⁰⁶ with expression influenced by maternal factors that include diet, psychosocial stress, history of allergic disease, and stage of lactation.¹⁰⁷ Several clinical studies have demonstrated an association between higher concentrations of TGF- β in milk and decreased risk of neonatal disease, including respiratory difficulty and allergy,¹⁰⁸ and a positive correlation between milk TGF- β concentrations and infant immunoglobulin production.¹⁰⁹ The presence of TGF- β attenuates the inflammatory response to cytokines IL-1 β and to the dsRNA viral mimic polyinosinic:polycytidylic acid in primary human fetal intestinal epithelium,¹¹⁰ and TGF- β in milk promotes the induction of antigenic tolerance during colonization of the neonatal intestine.¹¹¹

Exclusive enteral feeding of a preparation containing TGF- β to IL-10^{-/-} mice, a widely accepted model of spontaneous colitis, results in reduced expression of serum inflammatory markers, improved intestinal pathology, and the suppression of disease symptoms

compared with feeding of a control preparation to IL-10^{-/-} mice.¹¹² Dietary supplementation with TGF- β reduced the severity of inflammatory pathology in the colon and inhibited weight loss in wild-type mice treated with dextran sulfate sodium to induce colitis.¹¹³ The administration of formula containing TGF- β to allergy-prone Brown Norway rat pups suppressed characteristic type-1 hypersensitivity Th2 cytokines and enhanced a Th1-dominant pattern of cytokine expression, resulting in reduced mucosal mast cell activation compared with that observed in control pups fed standard formula. Importantly, these effects persisted after weaning.¹¹⁴ Cumulatively, animal studies of dietary TGF- β administration (summarized by Oddy and McMahon¹¹⁵) demonstrate potent protection from colitis and allergic disease.

The first formal evaluation of TGF- β supplementation in clinical practice reported the induction of disease remission over an 8-week period in 80% of pediatric patients with newly diagnosed Crohn's disease and in 58% of children with long-standing Crohn's disease.¹¹⁶ An independent follow-up study of a similar patient population reported a remission rate of 85% using an identical preparation.¹¹⁷ Compared with standard enteral nutrition formulas, TGF- β -supplemented formula is significantly more effective in inducing remission and reducing overall symptoms.¹¹⁸ In a recent study, TGF- β supplementation and traditional corticosteroid treatment were equally effective in inducing disease remission, although the TGF- β formula was associated with an improved nutrition profile and fewer side effects.¹¹⁹ This comprehensive TGF- β -containing enteral nutrition formulation is now available by prescription in the United States as a primary treatment for pediatric Crohn's disease or as complementary or alternative treatment of adult Crohn's disease (Modulen IBD, Nestlé Nutrition, Florham Park, New Jersey, USA). Although the mechanism of action in human patients has not been precisely defined, exclusive consumption of TGF- β -supplemented formula may be associated with alterations in the intestinal microbiota.¹²⁰ The supplementation of infant formula with TGF- β is an example of the potential use of bioactive milk components in clinical practice (Table 2).^{36–38,104,116–119,121–130}

APPLICATIONS OF HUMAN α -LACTALBUMIN MADE LETHAL TO TUMOR CELLS

The study of milk has resulted in unexpected discoveries, including the identification of a partially unfolded α -lactalbumin variant known as human α -lactalbumin made lethal to tumor cells (HAMLET). A peculiar multimeric form of α -lactalbumin isolated from human milk was found to induce apoptosis in transformed epithelium, embryonic cells, and lymphoid cells, sparing

Table 2 Clinical trials of systemic applications of human milk components

Milk component	Result of clinical trial	Reference(s)
Galacto-oligosaccharides	Reduction in incidence of infectious disease and allergy in some studies; meta-analysis indicates lack of efficacy	Moro et al. (2002) ⁶⁵ , Arslanoglu et al. (2007) ⁶⁶ , Westerbeek et al. (2011) ⁶⁷ , Mugambi et al. (2012) ⁶⁸
Bovine colostrum	Reduction in serum endotoxin following abdominal surgery	Bolke et al. (2002) ¹⁰⁴
TGF- β	80%–85% disease remission in pediatric Crohn's disease	Day et al. (2006) ¹¹⁶ , Navas-Lopez et al. (2008) ¹¹⁷ , Hartman et al. (2008) ¹¹⁸ , Soo et al. (2013) ¹¹⁹
HAMLET	Reduction in tumor size and increased apoptosis in bladder cancer	Mossberg et al. (2007) ¹²¹
Lactoferrin	90% resolution of skin papillomas Impaired growth of colon polyps	Gustafsson et al. (2004) ¹²² Kozu et al. (2009) ³⁷
PRP	Extended survival in lung cancer Stabilization or improvement of cognitive decline in Alzheimer's disease	Parikh et al. (2011) ³⁸ , Digumarti et al. (2011) ³⁹ Leszek et al. (1999) ¹²³ , Bilikiewicz & Gaus (2004) ¹²⁴
Casein peptides	Reduction in systolic and diastolic blood pressure in patients with hypertension	Sekiya et al. (1992) ¹²⁵ , Townsend et al. (2004) ¹²⁶ , Cadée et al. (2007) ¹²⁷ , Mizuno et al. (2005) ¹²⁸ , Turpeinen et al. (2011) ¹²⁹ , Ishida et al. (2011) ¹³⁰

Abbreviations: HAMLET, human α -lactalbumin made lethal to tumor cells; PRP, proline-rich polypeptide; TGF- β , transforming growth factor β .

mature epithelium.¹³¹ HAMLET is rapidly taken up by tumor cells and induces programmed cell death through a multifaceted mechanism. It accumulates in the nuclei, where it binds histones H3, H4, H2A, and H2B, and enhances histone acetylation, resulting in induction of cell death pathways and the insolubility of nuclear chromatin.¹³² In addition, interaction with the 20S proteasome leads to protease inhibition, prolonging the half-life of internalized HAMLET, and the induction of autophagy, dramatically increasing tumoricidal activity.¹²² Specific targeting of tumor cells remains an essential goal of modern cancer therapy. As a result of multiple complementary mechanisms, HAMLET exhibits broad *in vitro* antitumor activity in more than 40 lymphomas and carcinomas,¹³³ with minimal deleterious effects in matched, healthy, differentiated cell lines or in noncancerous tissues *in vivo*.¹²¹

The clinical application of HAMLET to the treatment of oncologic disease is currently under intense investigation. Therapeutic options for malignant tumors of the brain are extremely limited due to the necessity of preserving central nervous system tissue and the limited sensitivity of glioblastomas to chemotherapy.¹³⁴ Instillation of HAMLET into the brain tissue of rats with active human glioblastoma xenografts significantly delayed tumor progression and the onset of neurologic symptoms.¹³⁵ Similarly, instillation of HAMLET to the bladder of patients with bladder cancer prior to the resection of bladder tumors resulted in increased shedding of tumor cells in the urine, reduction in tumor size, and increased apoptosis in tumor biopsies but not in the adjacent healthy tissue.¹³⁶ A placebo-controlled study examined the topical application of HAMLET to human skin papillomas, a nonmalignant growth

of HPV-infected keratinocytes, reporting complete resolution of all lesions in >90% of HAMLET-treated patients, with no significant side effects.¹³⁷ This result was sustained for 2 years after treatment cessation in 83% of HAMLET-treated patients, a result that has been hailed as a breakthrough in the treatment of human skin papillomas.¹³⁸ Methods for the large-scale production of HAMLET may include the expression of recombinant proteins in microorganisms¹³⁹ or transgenic cattle with scale-appropriate isolation systems.¹⁴⁰ Thus, HAMLET represents a novel antitumor therapy – derived from the study of milk – with remarkable clinical potential.

ANTIEPILEPTIC PROPERTIES OF α -LACTALBUMIN

Alpha-lactalbumin is among the most abundant proteins in human milk. A complex containing α -lactalbumin and galactosyltransferase catalyzes the final step in lactose biosynthesis.¹⁴¹ Preclinical studies in animals indicate potential applications of α -lactalbumin in the treatment of epilepsy. Daily administration of α -lactalbumin over a course of 12 days significantly reduced the severity and duration of audible stimulus-induced seizure in genetically susceptible rats and protected against chemically induced seizures in mice. This effect was attributed to dramatic increases in serum tryptophan and subsequent improvement in serotonin-mediated functions.¹⁴² In another animal model of epileptogenesis, prophylactic α -lactalbumin administration reduced the spontaneous development of seizure in chemically treated rats. Bolus administration of α -lactalbumin during acute chemically induced seizure reduced seizure activity by as much as 90% in both rats and mice.¹⁴³ The tryptophan-rich protein structure of

α -lactalbumin may facilitate enhanced synthesis of serotonin from a tryptophan precursor.¹⁴² Russo et al.¹⁴³ have also suggested that α -lactalbumin inhibits *N*-methyl-D-aspartate receptors in the central nervous system, as evidenced by the reversal of α -lactalbumin-induced antiseizure activity during cotreatment with the *N*-methyl-D-aspartate antagonist D-serine. Epilepsy affects approximately 65 million individuals worldwide; well-tolerated antiepileptic agents based on milk α -lactalbumin may represent a treatment option for daily symptom management.

MULTIFUNCTIONAL CASEIN PEPTIDES

Casein peptides for treatment of cognitive decline

Originally isolated from sheep colostrum, proline-rich polypeptide is a low-molecular-weight milk protein fraction of 32 polypeptides consisting of an unusually high proportion of proline (22%) and nonpolar amino acids with sequence homology to β -casein and annexin.¹⁴⁴ During a preliminary trial of the putative immunomodulatory effects of proline-rich polypeptide, unexpected psychostimulation was observed in human subjects.¹⁴⁵ Subsequently, trials in rats demonstrated that administration of clostrinin, a specific proline-rich polypeptide preparation, promoted spatial learning and improved incidental memory,¹⁴⁶ leading to the proposal that proline-rich polypeptide attenuates neurologic decline. This hypothesis was evaluated in a double-blind, placebo-controlled study involving 46 Alzheimer's disease patients who received 100 μ g clostrinin or placebo every other day for 1 year and were evaluated at regular intervals by a blinded panel of psychiatrists. Compared with the control group, the clostrinin group showed a twofold increase in the incidence of disease stabilization or improvement.¹²³ These results prompted an independent group to conduct a double-blind, placebo-controlled, multicenter trial. Following 30 weeks of treatment with the proline-rich polypeptide preparation or placebo, clinical assessment scores indicated that the proline-rich polypeptide preparation had a significant effect in stabilizing both cognitive function and the ability to complete daily tasks, particularly among patients with less advanced disease at the start of the trial.¹²⁴ As a result of their demonstrable clinical effects in the treatment of Alzheimer's disease and their lack of a significant side-effect profile, proline-rich polypeptide preparations are now available worldwide as an over-the-counter nutraceutical dietary supplement (Clostrinin, ReGen Therapeutics Ltd., London, UK).

Molecular pathway analysis of mRNA expressed in cell cultures treated with proline-rich polypeptide

demonstrated altered expression of genes implicated in the synthesis of A β precursor protein and Tau phosphorylation and elevated expression of proteases that eliminate A β accumulation,¹⁴⁷ a primary target of drug design in Alzheimer's disease treatment.¹⁴⁸ In addition, proline-rich polypeptide enhanced defense against oxidative stress and suppressed inflammatory cytokine expression in vitro, suggesting that the observed benefits of proline-rich polypeptide administration in Alzheimer's patients are partially attributable to amelioration of inflammatory and oxidative damage.¹⁴⁷ Thus, in addition to the clinical benefits achieved through stabilization of Alzheimer's disease symptoms, current data suggest broad applications in neurologic and degenerative diseases.

Antihypertensive properties of casein peptides

Caseins make up a substantial portion of the total protein content of both human and bovine milk, accounting for as much as 50% of all dietary protein in breastfed infants. Although the majority of casein material in milk is digested and utilized as free amino acids, the proteolytic cleavage of casein by native or bacterial enzymes produces peptides with a wide variety of biologically relevant properties.¹⁴⁹ A diet rich in low-fat dairy products has been associated with antihypertensive effects in patients with stage I hypertension,¹⁵⁰ suggesting that bioactive milk components may contribute to improved blood pressure control. Early studies in rats prone to development of spontaneous hypertension and stroke examined the therapeutic effects of trypsin-digested milk casein preparations and succeeded in identifying 3 casein-derived peptides with antihypertensive activity. In vitro and in vivo experiments demonstrated that these peptides are potent inhibitors of angiotensin I-converting enzyme.¹⁵¹ The inhibition of angiotensin I-converting enzyme suppresses the generation of the peptide angiotensin II, a potent vasoconstrictor, from circulating angiotensin I substrate (reviewed by FitzGerald et al.¹⁴⁹).

Initial clinical trials of casein-derived peptides utilized undefined peptide pools. In the first preliminary clinical study of the antihypertensive effects of casein-derived peptides, daily consumption of 20 g of trypsin-digested casein resulted in a significant reduction in both systolic and diastolic blood pressure (mean: $-4.6/6.6$ mmHg) in 18 research subjects with mild hypertension but did not significantly alter blood pressure in healthy subjects.¹²⁵

A randomized, placebo-controlled prospective study evaluated the efficacy of a single dose of a defined casein peptide (amino acid sequence: FFVAPFEVFGK) in 10 hypertensive research subjects, reporting

significant reduction in diastolic blood pressure at 6 hours postgestion.¹²⁶ A double-blind, placebo-controlled follow-up study in which 48 volunteers were given a daily dose of 3.8 g of the FFVAPFEVFGK casein peptide for 4 weeks demonstrated a significant reduction in both systolic and diastolic blood pressure (mean: −10.7/6.9 mmHg) as well as a reduction in plasma angiotensin II and aldosterone compared with the placebo-treated group.¹²⁷ This peptide, frequently referred to as C12, is currently available as an over-the-counter nutraceutical preparation sold worldwide under a variety of trade names by DMV International (Veghel, The Netherlands), although its specific clinical utility relative to other widely available antihypertensive drugs has yet to be established. Another defined casein hydrolysate preparation consisting of two casein tripeptides, valine-proline-proline (Val-Pro-Pro) and isoleucine-proline-proline (Ile-Pro-Pro), has been demonstrated to produce significant reductions in systolic and diastolic blood pressure in a large cohort of patients with mild hypertension.¹²⁸ Recently, this result was independently

validated in a distinct population.¹²⁹ Val-Pro-Pro/Ile-Pro-Pro preparations are remarkably well tolerated at up to 5 times the effective dose.¹³⁰ Recent data indicate that human milk casein peptides are generated *in vivo*, and the analysis and biological characterization of these structures may result in the identification of casein peptides with enhanced activity.¹⁵²

CONCLUSION

The study of human milk has resulted in abundant opportunities for translational medicine (Tables 1 and 2; Figure 1). Several milk-derived therapeutic preparations are already available to clinicians. Among the most notable are TGF- β -supplemented formula, which has become an important treatment option for some types of pediatric inflammatory bowel disease,¹¹⁹ and hyperimmune bovine colostrum preparations used in the prevention of enterotoxigenic *E. coli* infection.¹³ Many human milk components have shown promise in preclinical studies and are undergoing active clinical evaluation. In

Figure 1 Diagram illustrating the wide range of applications for bioactive milk compounds in the treatment of human disease. Molecules undergoing preclinical evaluation are listed on the left side of the diagram underneath each disease model or clinical application under consideration. Milk components undergoing active clinical evaluation or currently in clinical use are listed on the right side of the diagram underneath each human disease in which clinical efficacy has been demonstrated. *Abbreviations:* GAG, glycosaminoglycan; HAMLET, human α -lactalbumin made lethal to tumor cells; HMO, human milk oligosaccharide; IBD, inflammatory bowel disease; NEC, necrotizing enterocolitis; PRP, proline-rich polypeptide; sIgA, soluble immunoglobulin A.

other cases, so-called nutraceutical products, which exhibit mild clinical effects but virtually no measurable toxicity, have been developed. A final category includes emerging compounds for which in vitro experiments and animal models indicate remarkable bioactivity and potential therapeutic utility, such as human milk oligosaccharides and glycosaminoglycans.^{29,84} Taken together, the translation of milk biology into effective clinical therapies has proven to be a remarkably rich area of research.

A review of these efforts suggests a relatively conserved pathway for the translation of bioactive milk compounds to clinical applications. The use of bovine milk products represents the most direct path to widespread application, although the continued study of the structure and function of human milk components will likely identify structures unique to human biology. The isolation and biochemical characterization of individual milk components can define the bioactive milk matrix in terms of specific structural properties. This information can, in turn, guide the evaluation of biological function in vitro and in animal models of human disease, in many cases using structural data on bioactive compounds in milk to make analogies to other areas of research that suggest potential biological function in a milk-specific context.

In some cases, such as the development of casein-derived antihypertensive agents,¹⁵⁰ epidemiologic data associating breastfeeding or dairy consumption with specific health benefits or disease protection may inform the evaluation of biological function. Indeed, epidemiologic data suggests that milk consumption is associated with protection from many of the most widely prevalent human diseases.^{2–4,153} Given the tremendous range of biological activity within the milk matrix, accurate attribution of specific biological functions to specific structures requires defined preparations with minimal influence from undefined “contaminants.” A small subset of trials have suggested that multiple compounds acting through disparate mechanisms may exert compounding protective effects, and future investigations should evaluate the coordinated application of defined multicomponent therapies derived from milk.^{25,27} Finally, the attribution of specific biological properties to defined milk structures enhances the case for patent protection. This is an important consideration when seeking industrial investment in translational efforts, and defined preparations have an advantage over nutraceutical preparations with undefined milk structures, which are generally not subject to patent protection in the United States.¹⁵⁴

Once a specific structure has been identified and its biological function characterized in relevant animal models of disease, however, a significant hurdle often

slows progress towards clinical application, namely, development of techniques for large-scale production. For example, preclinical research on lactoferrin and human milk oligosaccharide has followed a roughly similar trajectory over the past 2 decades,^{30,84} but the production of recombinant human lactoferrin has accelerated the clinical evaluation of therapeutic applications in cancer and infectious disease, while means of large-scale production of human milk oligosaccharides have only recently been devised, so that clinical data is still forthcoming. Finally, clinical trials must be conducted in appropriate patient populations, guided by preclinical data in animal models of disease. In clinical trials, a significant advantage of milk-derived bioactive components over other pharmaceutical products is the generally excellent tolerability of milk compounds.^{39,68,126} The remarkably safe side-effect profile of many milk-derived compounds increases patient participation and minimizes clinical and financial risk to human subjects and host institutions. A pathway for the translation of milk bioactive components to clinical applications may, thus, be outlined as follows: 1) isolation and biochemical characterization; 2) preclinical testing in vitro and in relevant animal disease models; 3) large-scale synthesis or purification from animal sources; and 4) design of appropriate clinical trials guided by preclinical data.

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