

REVIEW ARTICLE

Lactoferrin, a Pleiotropic Protein in Health and Disease

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Abstract

Significance: Lactoferrin (Lf) is a nonheme iron-binding glycoprotein strongly expressed in human and bovine milk and it plays many functions during infancy such as iron homeostasis and defense against microorganisms. In humans, Lf is mainly expressed in mucosal epithelial and immune cells. Growing evidence suggests multiple physiological roles for Lf after weaning. **Recent Advances:** The aim of this review is to highlight the recent advances concerning multifunctional Lf activities. **Critical Issues:** First, we will provide an overview of the mechanisms related to Lf intrinsic synthesis or intestinal absorption as well as its interaction with a wide spectrum of mammalian receptors and distribution in organs and cell types. Second, we will discuss the large variety of its physiological functions such as iron homeostasis, transportation, immune regulation, oxidative stress, inflammation, and apoptosis while specifying the mechanisms of action. Third, we will focus on its recent physiopathology implication in metabolic disorders, including obesity, type 2 diabetes, and cardiovascular diseases. Additional efforts are necessary before suggesting the potential use of Lf as a diagnostic marker or as a therapeutic tool. **Future Directions:** The main sources of Lf in human cardiometabolic disorders should be clarified to identify new perspectives for future research and develop new strategies using Lf in therapeutics. *Antioxid. Redox Signal.* 00, 000–000.

Introduction

LACTOFERRIN (LF) WAS DISCOVERED in 1939 as an iron-containing protein in bovine milk (189). However, initial studies of this protein did not really start until ~1960 when technological progress made it possible to properly extract it from human and bovine milk while achieving its full characterization (82). Since then, more than 7000 articles have been published and the findings have clearly established its multiple functions at the physiological, cellular, and molecular levels. The best-known Lf property is its strong iron-binding ability. This led to the hypothesis of its implication in iron transportation and metabolism. However, Lf function is not limited to iron homeostasis because Lf can display strong antimicrobial activity. Importantly, its localization on the mucosal surface represents one of the first defense systems against microorganism invasion (80).

Subsequent studies have extended the features of Lf to the regulation of transcriptional activity and morphogenesis of some tissues. Lf also seems to be implicated in a spectrum of physiopathological events related to oxidant and inflammatory processes, as well as carcinogenesis and energy metab-

olism. The aim of this review is to summarize and update the major roles attributed to Lf. We will discuss the potential mechanisms contributing to its various actions. We will develop not only its endogenous role but also its implication in the etiopathology of oxidative stress (OxS) and metabolic disorders. Similarly, the use of exogenous Lf and derived peptides as therapeutic agents will be examined to identify new perspectives for future research.

Lf Structure

Lf is an 80-kDa glycoprotein with high iron affinity. Due to its homology of sequence with serum transferrin, Lf is classified as a member of the transferrin family. This family of nonheme iron-binding proteins is characterized by critical roles played by anions in iron binding (97). In most cells, Lf is secreted in its iron-free form (apo-Lf) and part of this Lf will bind to ferric irons (Fe^{3+}) until saturation (holo-Lf) (64). The holo-Lf and apo-Lf adopt “closed” and “open” forms, respectively.

The sequence and the conformation of Lf have been described (Table 1) (79). Lf displayed a structural organization

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TABLE 1. PHYSICO-CHEMICAL PARAMETERS OF HUMAN LACTOFERRIN AND DELTA-LACTOFERRIN

Gene <i>LTF</i>	Location: 3p21.31 Exon count: 18	
Protein <i>Lactoferrin</i> (PO2788-1)	Number of amino acids: 710 Molecular weight: 78.182kDa Theoretical pI: 8.47	
	Prostranslational modifications	
	Active sites	
	Sequence	156-497-642 79-111-211-272-414-454-547-616 136-140-142-143-480-484-486-487
		Glycosylation: Metal binding amino acids Carbonate binding amino acids MKLVFLVLLFLGALGLCLAGRRRSVQWCAVSQPEATKCFQWRNMRKVRGPPVSCIKRDSPIQCIQAIA ENRADAVTLDGGFIYEAGLAPYKLRPVAAVYGTERTQPRTHYAVAVVKKGGFQNLQGLKSCHT GLRRTAGWNVPIGTLRPFLNWTGPPEPIEAAVARFASCVPGADKGGQFPNLCRLCAGTGENKCAFSS QEPYFSYSGAFKCLRDGAGDVAFIRESTVFEDLSDEAERDEYELLCPDNTTRKPKDKFKDCHLARVPSHA VVARSVNGKEDAIWLLROAQEKFGKDKSPKQFLFGSPSQKDLLFKDSAIQFVSRVPPRIDSGLYLG SGYFTAIQNLKSEEEVAAARRARVWCAVGEQELRKCNCQWVSLSEGSVTCSSASTTEDICIALVLKGE ADAMSLDGGYVYTAGKCGLVPVLAENYKQQSSDPDNCVDRPVEGYLAVAVRRSDTSLTWNSV KGGKSCHTAVDRTAGWNIPMGLLNFQTSCKFDEYFQSCAPGSDPRSNLALCALCIGDEQGENKCVPN SNERYGYTGAFRCLAENAGDVAFVKDVTVLQNTDGNNEAWAKDLKLAADFALLCLDGGKRRKPVTE ARSHLAMAPNHA VVSRMDKVERLKQVLLHQAKFGRNGSDCPDKKFLQSETKNLL FNDNTECLARLHGKTTYEKYLGPQYVAGITNLKCKCSTSPLEACEFLRK
	Composition (Mole%)	Lysine (6.44); Histidine (1.19); Arginine (5.49); Aspartic acid (10.84); Threonine (5.32); Serine (6.70); Glutamic acid (10.41); Proline (4.30); Glycine (7.71); Alanine (8.88); Half cysteine (4.49); Valine (7.28); Methionine (0.21); Isoleucine (2.78); Leucine (8.23); Tyrosine (2.79); Phenylalanine (3.96); Tryptophan (2.94)
<i>Delta-Lactoferrin</i> (PO2788-2)	Number of amino acids: 666 Molecular weight: 73.161 kDa Theoretical pI: 8.20	
	Prostranslational modifications	
	Protein sequence orthology (% identity)	Phosphorylation/o-GlcNaeylation Serine-10
	<i>Pan troglodyte</i> (98%)	
	<i>Mus musculus</i> (71%)	
	<i>Rattus norvegicus</i> (62%)	
	<i>Bos Taurus</i> (70%)	
	<i>Ovis aries</i> (71%)	
	<i>Capra hircus</i> (71%)	
	<i>Equus caballus</i> (75%)	

The different parameters are extracted from NCBI database (gene ID: 4057) and from Uniprot protein database (PO2788).

shared among members of the transferrin family. Lf is the youngest member of the transferrin family and appeared after the placental/marsupial split around 125 million years ago (98). Lf is formed by a single polypeptide chain and folded into two globular lobes (Fig. 1). These lobes are, respectively, called the N-terminal and C-terminal lobes based on their localization. Each lobe contains two domains (referred to as N₁ and N₂, or C₁ and C₂) that enclose a deep cleft within the iron-binding site. The two lobes have a notable degree of homology because they originate from the duplication of a 40-kDa protein (98).

Humans Lf (hLf) may have multiple post-translational modifications such as phosphorylation (169) and enrichment with N-glycans (11) (Table 1). Although the exact function of these modifications is not fully understood, they may protect Lf from proteolysis and are also involved in receptor recognition (11, 237).

Different isoforms of human 80-kDa Lf have been identified. Lf- α is the classical iron-binding form, while Lf- β and Lf- γ are not able to bind iron but they have ribonuclease activity (58). So far, there is little information about the structural differences between these isoforms, they, however, seem to have similar physicochemical properties. These isoforms, which were first isolated from human milk, are also detected in human granulocytes (57). There is also an intracellular truncated isoform of Lf described in humans and called delta-Lf (Fig. 1 and Table 1) (183). This isoform is induced by the activation of an alternative promoter that leads to the replacement of exon 1 by exon 1 β and to the translation to a 73-kDa protein that lacks the leader sequence and the first 26 amino acid residues (Fig. 1D). Since the Lf N-terminus (involved in certain Lf functions) is removed, delta-Lf acts differently from the classical isoform. Finally, recent studies

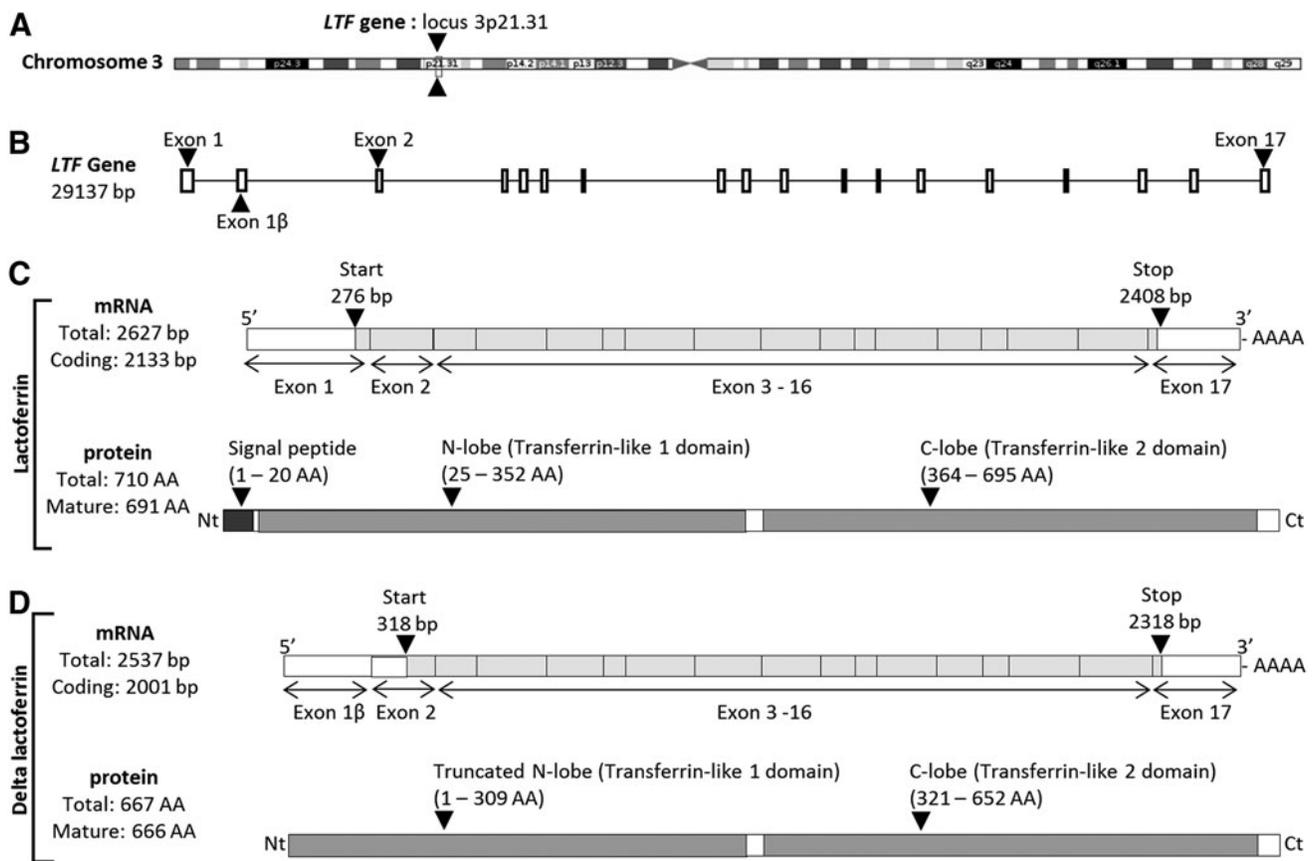


FIG. 1. From gene to protein: schematic representation of lactoferrin and its isoform synthesis in human. (A) Representation of human chromosome three containing *lactoferrin* gene located at locus 3p21.31. (B) Illustration of human *lactoferrin* gene containing the 18 exons necessary to produce lactoferrin and delta lactoferrin. Exons are represented by white boxes. Introns located between exons are represented by black lines. The exons 2 to 17 are common to *lactoferrin* and *delta lactoferrin*. (C) Representation of the synthesis of human lactoferrin. The *lactoferrin* mRNA is composed of exon 1 to 17 and does not contain exon 1 β . Coding sequence is represented in gray on mRNA sequence. The *lactoferrin* traduction starts at position 276 bp in exon 1 and leads to a preprotein of 710 AA containing a signal peptide for extracellular secretion. Mature protein has 691 AA and contains 2 transferrin-like domain named, respectively, N-lobe and C-lobe. (D) Illustration of the synthesis of human delta lactoferrin. *Delta lactoferrin* mRNA is composed of exon 1 β to 17 except exon 1. *Delta lactoferrin* traduction starts at the position 318 bp of the exon 2 and leads to a cytosolic protein of 666 AA that does not contain a signal peptide. The first transferrin-like domain of *delta lactoferrin* is truncated by 19 AA. Gene, mRNA, and protein are represented at scale using information from NCBI database (NG_023257.1; NM_002343.4; NM_001199149.1) and from Uniprot protein database (PO2788). Representation of human chromosome three containing *lactoferrin* gene is extracted from UCSC Genome Bioinformatics database. AA, amino acids; bp, base pair.

have shown that delta-Lf may act as a transcriptional activator in cell death regulation (121, 143).

Sources, Synthesis, and Regulation of Lf

Lf is the second most abundant protein in human milk. It is mainly present in bodily fluids since it is found in most mucosal secretions such as saliva, tears, bile, pancreatic juice, intestinal mucus, seminal fluid, and genital secretions (5). The synthesis and secretion of Lf by the exocrine glands are constant. Nevertheless, Lf synthesis may be regulated by prolactin in the mammary gland (140) and by estrogens in the reproductive organs (162). The responsiveness of Lf gene to estrogen is mediated through an estrogen response element (192). In the endometrium, the synthesis of Lf is also influenced by the epidermal growth factor (141). Therefore, it is not surprising that plasmatic Lf levels vary not only during the menstrual cycle but also during pregnancy (29, 197). Lf secretion in human milk is inversely correlated with the day after parturition (73). Although a decrease of Lf content is observed, the iron saturation of Lf is increased leading to a stable iron content during lactation (73). In addition, hLf glycosylation displays dynamic changes during lactation. Collectively, these changes affect bacterial binding to epithelial cells and thus inhibit pathogen adhesion (11).

Lf is also expressed in some organs. For example, human kidneys synthesize Lf that is secreted throughout the collecting tubules. It may also be reabsorbed in the distal part of the tubules (1). Lf is also present at low concentrations in the blood circulation. Its origin is not fully understood, but it seems to be predominantly derived from polynuclear neutrophil degranulation during inflammation. Lf is synthesized during the differentiation of neutrophils and afterward mainly stored in secondary granules (175). However, studies do not necessarily show a correlation of plasmatic Lf and neutrophil count (16, 151). Therefore, additional efforts are still necessary to clearly determine the cellular and tissular origin of circulating Lf.

Finally, Lf gene expression may be affected by epigenetic regulation. Notably, the methylation of the promotor and the first intronic region of Lf has been described in the pathophysiology of cancer (77, 203).

Digestion, Transport, and Metabolism of Lf

The metabolism of Lf is quite interesting since Lf may display different actions in the body according to its endogenous and exogenous origins. Breastfeeding is an example of exogenous Lf intake in infants. After weaning, babies may continue to have an exogenous supply of Lf mainly through the consumption of milk from cows or other species. Recently, the European Food Safety Authority has approved the use of bovine Lf (bLf) as an ingredient in manufacturing food products (45). Consequently, it is necessary to understand Lf digestion, transport, and metabolism to comprehend its physiological impact.

The origin and form of Lf may influence its digestibility. hLf is more resistant to gastric proteolysis than bLf (22, 212). Iron-saturated or holo-Lf is more resistant to proteolysis than apo-Lf (190). Different studies have assessed the gastrointestinal survival of Lf in humans. Although intact Lf and its functional fragments have been observed in infants and adults (65, 205), both treat Lf differently in the digestive process. In

fact, the presence of intact Lf was detected in the feces of breast-fed babies, but its survival decreased with increasing age (190). Studies of other species confirmed these results. For example, Lf degradation is lower than casein digestibility in the small intestine of suckling pigs but not in adults (44). Interestingly, the gastric hydrolysis and intestinal luminal degradation of Lf are higher in weanling than in suckling rats, suggesting there may exist different mechanisms of Lf digestion through life (23).

Recent data have shown that hLf is not degraded by proteases contained in milk. Its digestion rather starts in the baby's stomach and generates many functional peptides that are biologically available in the proximal intestine (36). In addition, as digestive proteolysis is not totally effective at this stage in babies, a transfer of intact Lf from the intestinal lumen to infant blood might occur, as was reported earlier for other proteins (26).

In adult humans, 60–80% of orally administered bLf survives after gastric digestion (Fig. 2) (205). Conversely, the same authors in another study failed to detect orally administered recombinant hLf in ileostomy patients, suggesting that Lf is digested in the upper gastrointestinal tract and could not reach the distal small intestine and colon (204). However, two points need to be considered. First, the authors used a recombinant protein that may not possess the strategic pattern of post-translational modification that is necessary to protect Lf from digestion (212). Second, the authors detected a faint excretion of Lf over time, signifying an endogenous production of Lf by the gastrointestinal tract. Finally, these investigators do not take into account the fact that Lf is not generally consumed alone and accompanying compounds such as lipids may protect it from proteolysis. A recent study has even shown that Lf, encapsulated in different lipid-based delivery systems, may undergo a strongly decreased degradation during digestion (230).

It is also interesting to consider that Lf peptide generated by proteolysis may be absorbed and may even induce biological functions (Fig. 2). Experiments have emphasized that this proteolysis generates peptide fragments capable of conserving the biological activity such as lactoferricin (Lf_{cin}) (18). Some authors suggest that a similar process may occur during digestion and may participate in the bioactivity of orally administered Lf (36). Indeed, recent experiments have suggested that these peptides play a beneficial role in microbial defense and cancer (232, 233). So far, the biological functions and mechanisms of action of these peptides are not fully understood.

In vitro studies suggest that hLf and bLf may target enterocytes and exert cell proliferation and differentiation (3, 111). At this epithelial level, Lf binds to the intelectin receptor and is internalized *via* a clathrin-dependent pathway (81). A transfer of Lf from the intestine into blood in adults has also been suggested since bLf is absorbed by intestinal epithelial cells and transported into the blood circulation *via* the lymphatic pathway when infused in the rat's duodenum (199). These authors have suggested that this mechanism prevents immediate metabolism of Lf by the liver. Other studies support these findings. The presence of bLf has been detected in blood samples 4 h after ingestion of enteric-coated Lf in humans (180). Enteric-coated bLf administered to rats is also rapidly found in mesenteric fat, suggesting that exogenous Lf may exert bioactivity directly on adipose

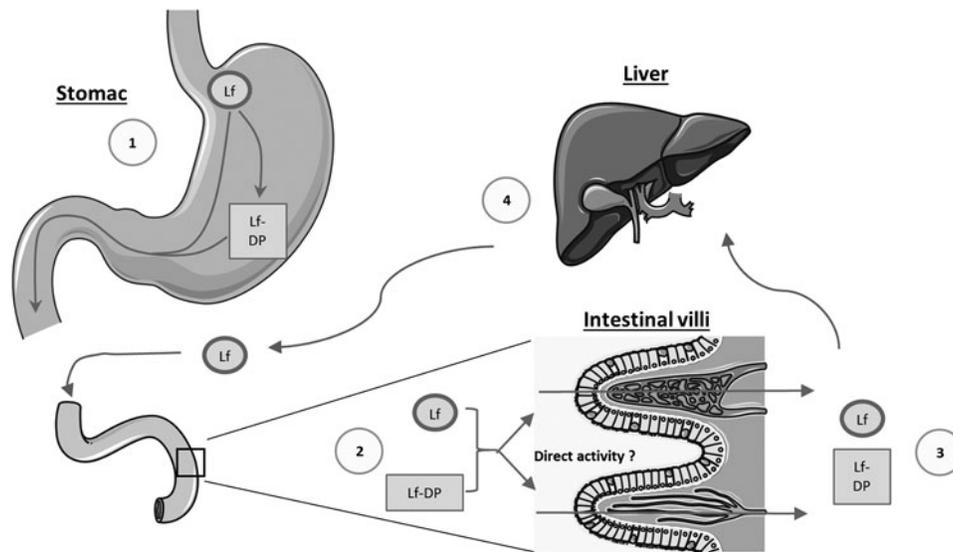


FIG. 2. Schematic representation of putative exogenous lactoferrin digestion and metabolism. (1) After ingestion, a part of Lf is proteolysed by gastric enzyme that generates Lf-DP. (2) Both Lf and Lf-DP may interact with the intestinal intelectin-1 receptor. They can also be absorbed through enterocyte by endocytosis and reached the lymphatic and blood circulation. (3) At this level, both Lf and Lf-DP may interact with specific receptors and exert their physiological actions. (4) However, a part of Lf may integrate the enterohepatic circulation and may be secreted in the intestine. *Arrows* represent the enterohepatic pathway. The Figure was made with Servier Medical Art. Lf-DP, lactoferrin-derived peptide.

tissues (153). In addition, pharmacokinetic studies in mice have documented that ingested bLf rapidly accumulates not only in the liver, spleen, and kidney but also in the brain, suggesting a transfer mechanism through the blood–brain barrier (53). Overall, these data suggest the potential role of Lf in different organs and especially in the central nervous system, which requests thorough elucidation. Finally, studies in animals have shown that Lf may be transported into the circulation from the intestinal lumen and then excreted into the bile, thereby suggesting an enterohepatic circulation of Lf (68, 168). The potential role of Lf in the enterohepatic circulation is not fully understood but it may be useful for iron homeostasis or in the regulation of intestinal inflammation mechanisms.

Finally, some aspects should be clarified soon. What is the role of intestinal microflora in Lf digestibility and absorption and alternately how does Lf impact on microbiota profiling? For the moment, studies have demonstrated that Lf decreases the growth of some bacteria such as *Escherichia coli* and *Salmonella spp.* and promotes the development of the *Bifidobacteria* strain (75, 124, 148, 194); however, it is important to mention the intriguing absence of data concerning the impact of gut bacteria on Lf metabolism and bioavailability.

Interactions with Mammalian Receptors

Despite its homology with transferrin, Lf cannot bind to the transferrin receptor, but it has a strong affinity to a number of receptors from many unrelated protein families (Table 2).

Omentin-1

Omentin was first identified as a lectin that recognizes galactofuranosyl residues (207). It is a glycoprotein of 35 kDa that may form a 120-kDa homotrimer in which polypeptides are bridged by disulfide bonds (Table 2). Omentin exists in a circulatory form that is synthesized by visceral adipose tissue

(40), but there is also a membrane form of omentin-1, named intelectin-1, able to bind Lf (195). This form does not possess a transmembrane region, but rather a GPI anchor that allows binding to hLf specifically on the apical membrane of the human infant intestine (195). At this level, omentin-1 seems to be expressed by Paneth and goblet cells, but the major expression of omentin occurs in the enterocyte where it is localized in lipid raft domains (225).

The intestinal function of this receptor is not clear. As a lectin, it can bind to galactofuranose, a carbohydrate found in bacterial cell walls, suggesting an involvement in the defense against pathogens. However, so far there are no data indicating that the Lf-intelectin interaction is implicated in this specific process (207). It has been reported that these receptors are implicated in the endocytosis of Lf *via* a clathrin-mediated pathway and they can trigger intracellular signaling (81). The human intelectin receptor can also bind and internalize bLf, while displaying bioactivities (111). Interestingly, recombinant human omentin-1 exhibits a higher binding ability to bLf than to hLf. Hydrolysate of bLf can also bind to this receptor (181). Apparently, there is no interaction between the circulating form of omentin-1 and Lf. Circulating omentin-1 is now known as an adipocytokine associated to metabolic diseases such as obesity (40) and type 2 diabetes (T2D) (229). Omentin-1 is inversely related to obesity (40) and is increased after weight loss (131). It will be interesting to clarify whether Lf modulates omentin-1 metabolic activities. Similarly, additional work is necessary to determine whether Lf from endogenous and exogenous origins modulates omentin-1 expression in visceral adipose tissue.

Low-density lipoprotein receptor-related proteins

Low-density lipoprotein (LDL) receptor-related proteins (LRP) are members of the LDL receptor family that also

TABLE 2. PRINCIPAL CHARACTERISTICS OF THE MAIN LACTOFERRIN RECEPTORS IN HUMAN

Receptor	Main site expression	Subcellular localization	Ligands	Binding to			Physiological functions	References
				Human Lf	Bovine Lf	Lf-derived peptides		
Intelectin-1	Small intestine, Colon, Visceral adipose tissues	Plasma membrane, Exist in a soluble form	Galactofuranosyl residues	Yes	Yes	Yes	Defenses against pathogen (putative role), Increase insulin sensitivity (soluble form)	(40, 195, 207, 225)
LRP1	Liver, Adipose tissues, Testis, Endometrium, Lung, Brain	Plasma membrane	Multiligand receptor (lipoproteins, extracellular matrix proteins, protease/protease inhibitor complexes, virus, growth factors, and cytokines)	Yes	Yes	NI	Endocytic receptor for apoptotic cells, Plasma clearance of chylomicron remnants	(35, 66, 126, 128)
LRP2	Absorptive epithelia mainly, kidney	Plasma membrane	Multiligand receptor (carrier proteins, peptides, hormones, signaling molecules, enzymes, immune-related protein)	Yes	NI	NI	May mediate HDL endocytosis, Potential endocytic receptor	(35, 122, 125, 154)
Nucleolin	Ubiquitous	Plasma surface, cytoplasm, nucleus	Endostatin and other potential ligands	Yes	NI	NI	Chromatin decondensation by binding to histone H1play a role in transcription and ribosome assembly	(103, 129, 191, 198)

Main site expression and subcellular localization for each receptor are extracted from the Human Protein Atlas database. Lf, lactoferrin; NI, none identified.

includes LDL and VLDL receptors. Only LRP1 and LRP2 are known to bind to Lf (Table 2) (35, 154). LRP1 is composed of two subunits. The 515-kDa α chain resides entirely in the extracellular space and is bound noncovalently to the 85-kDa β chain (126). LRP1 is not a specific for Lf since it recognizes more than 40 ligands such as lipoproteins, extracellular matrix proteins, protease/protease inhibitor complexes, viruses, growth factors, and cytokines, suggesting that this receptor plays diverse biological roles in cell growth, migration, and lipid metabolism. LRP2 is also a multiligand receptor expressed at the apical surface of epithelial borders and it internalizes a variety of ligands, including hormones, signaling molecules, and nutrients (122).

Lf binding to LRP mediates specific biological functions. As only limited information is available concerning Lf actions on LRP2, we will thus focus only on LRP1. The hepatic clearance of plasmatic Lf is mediated by binding to LRP1 (128). This receptor also triggers a cellular response to Lf stimulation. For example, Lf induces a mitogenic response in osteoblastic cells through its binding to LRP1 (Table 2) (66). Using LRP receptors, Lf may take part in the regulation of lipoprotein metabolism. Intravenous injection of Lf has been shown to inhibit the LRP-mediated uptake of apolipoprotein E-containing lipoproteins such as VLDL and chylomicron remnants (34). Unfortunately, the lack of information does not allow us to draw definitive conclusions about the implication of the Lf/LRP complex in lipid metabolism. However, recent observations have shown that bLf administration to mice reduced hepatic triglyceride (TG) levels (138). Evidently, much work is requested to clarify the function of Lf/LRP in lipid metabolism essentially following its influence on hepatic uptake of lipoproteins.

Nucleolin

Nucleolin is a ubiquitous protein that is highly conserved in vertebrates. This 77-kDa protein is mainly localized in the nucleolus where it constitutes 10% of proteins (Table 2) (129). *Nucleolin* is composed of three domains: an N-terminal domain rich in acidic residues, a central globular domain with four RNA-binding domains, and a C-terminal domain with numerous glycine-arginine-rich motifs (129). Despite its predominant localization in the nucleolus, a part of nucleolin is also found in the cytosol and cell surfaces. At the nucleolus level, nucleolin mainly participates in ribosomal biogenesis, chromatin remodeling, and the nucleocytoplasmic transport of newly synthesized pre-RNAs (198). At the cytosol level, this protein is used in ribosomal assembly and it enhances the stability of some mRNA. For example, nucleolin binds to a specific sequence of the 3'UTR of *Bcl2* mRNA leading to the inhibition of ribonuclease degradation (224). The expression of nucleolin at the surface of cells is surprising given that its sequence contains a nuclear localization sequence without peptide signal for its secretion. At this level, nucleolin may act as a coreceptor of numerous ligands and participate in their endocytosis and nuclear trafficking (103, 188). Once secreted, nucleolin seems to be associated with lipid rafts. This organization is dependent on the actin cytoskeleton and may take part in the endocytosis of ligands (74).

Some experiments have shown that surface nucleolin is able to bind and internalize Lf with moderate affinity

(approximately 240 nM) (4, 103). The nucleolin-binding site is located on both the N- and C-terminal lobes of Lf. However, only the C-terminal region of Lf is necessary for its binding (103). After binding to the cell surface, Lf is internalized and appears in the nucleus where it is found attached to DNA. Therefore, nucleolin may represent a candidate protein for nuclear traffic and transcriptional action of Lf. Nucleolin can bind to apolipoproteins B- and E-containing lipoproteins in HepG2 cells (177). New experimental approaches are needed to highlight its exact role in liver lipoprotein metabolism and to determine its eventual participation in the regulation of lipoprotein metabolism *via* surface nucleolin receptors.

Other Lf-interacting proteins

Lf also interacts with other proteins that may mediate its actions. Rawat *et al.* have shown that the multifunctional glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may be a novel Lf receptor in macrophages (167). In fact, GAPDH is expressed at the surface of macrophages where it binds to Lf and even shuttles it to the endosomal compartment. Upon iron depletion, macrophages increase Lf binding enhancement of surface GAPDH expression without modulating the expression of other Lf receptors such as LRP.

Findings on this recently discovered receptor suggests that Lf action may be subtly regulated by the expression of various receptors in target cells. Early investigations have reported that Lf can interact with monocyte differentiation antigen CD14, a coreceptor of the MD-2/Toll-like receptor 4 complex implicated in the innate immune response to bacterial lipopolysaccharides (LPS). Lf may also bind to other proteins such as ceruloplasmin (186), osteopontin (228), and tear lipocalin (61). These interactions probably stimulate Lf activity, thereby influencing processes such as OxS. Accordingly, Lf can form a complex with the copper transporter ceruloplasmin that limits its destruction in the presence of hydrogen peroxide and the release of copper ions that amplify OxS (186). Altogether, these data emphasize the complex regulation of Lf properties by different receptors and interacting proteins, which allow triggering of Lf functions in line with cellular needs and environment status.

Lf acting as a transcription factor

Lf action is not limited to its receptor-mediated response. Indeed, after binding at the cell surface, Lf may be internalized, targeted to the nucleus, and bind to DNA (60). Lf may recognize the specific sequence of DNA and the subsequent interaction may lead to transcriptional activation (71). In this context, the human *interleukin (IL)-1 β* gene contains five putative Lf binding sites in its 5'-flanking sequences allowing the transcription of this gene (187). In contrast, Lf binding to the granulocyte macrophage colony-stimulating factor promoter may downmodulate the Lf activity in specific conditions (160).

The mechanism of DNA binding by hLf is not fully understood and seems to implicate various processes. Lf possesses two DNA-binding domains with different affinities for DNA (85). These DNA-binding domains are located in the N-terminus domain of Lf. The latter is known to be highly basic and may, in part, contribute to the DNA interaction

through the binding of phosphate to DNA. This hypothesis is validated by the use of polyanionic ligand-like heparin that inhibits this interaction (85). Furthermore, hLf may contain nuclear localization sequences. Studying the transcriptional activity of delta-Lf, Mariller *et al.* have not only identified two delta-Lf-responsive elements on the *Skp1* gene promoter but also a nuclear localization sequence present in both delta-Lf and full-length hLf (121). Other groups reported that the GRRRR sequence localized in the N-terminal domain of hLf is also a nuclear localization sequence (161). Finally, nuclear internalization of Lf is not limited to the specificity of its sequence. For example, GFP-tagged Lf expressed by cells is present in the cytoplasm but not in the nucleus (108). These data suggest that other actors such as Lf receptors may be implicated in this process. As mentioned previously, nucleolin may represent a good candidate to shuttle Lf into the nucleus. hLf and intelectin-1 receptors are internalized in Caco-2 cells and internalized in the nucleus. Using a chimeric protein of Lf, the investigators have also identified the importance of the first nineteen residues of Lf in this phenomenon (196). The nuclear internalization of Lf and its binding to DNA are influenced in certain conditions. For example, the C-terminal domain of hLf contains an ATP-binding site that is involved in the dissociation of Lf into monomers in the presence of ATP, thereby leading to a better affinity of Lf to oligonucleotides (178). Alternatively, breast cancer cells gain the capacity to take up bLf only in the presence of retinoic acid (12).

In conclusion, strong evidence suggests that exogenous Lf can be internalized in the nucleus, followed by binding to DNA and directly acting as a transcriptional activator. However, the mechanisms of this phenomenon request a thorough elucidation to fully understand Lf importance in regulating cellular functions and physiological roles. Numerous issues related to potential cooperation between Lf and other transcription factors need to be clarified. This phenomenon has so far been well described for hLf, but there is no information about the transcriptional action of other forms of Lf.

Physiological Roles of Lf and Mechanisms of Action

There is growing evidence that Lf is a pleiotropic protein that can display multiple physiological actions (Fig. 3). Accordingly, this protein is involved in different pathologies.

Iron homeostasis, transportation, and sequestration

Red protein from bovine milk was first isolated in 1939, but it took two decades to discover that this coloration is linked to Lf ability to bind to Fe³⁺ (130, 189). Structural studies have shown that each lobe of Lf contains an iron-binding site. These lobes are divided into two domains that seem to adopt two conformational states depending on their association with metal ions. In apo-Lf, these domains are in open state and form a deep cleft that makes it possible to fix Fe³⁺ owing to the presence of specific amino acids (two tyrosines, one asparagine, and one histidine) surrounding it (10). In addition, binding requires the presence of a carbonate ion that stabilizes Fe³⁺. This phenomenon induces conformational changes in which the two domains adopt a relative rotation around a hinge at the back of the iron binding site and close over the bound metal ion (79). The interactions between

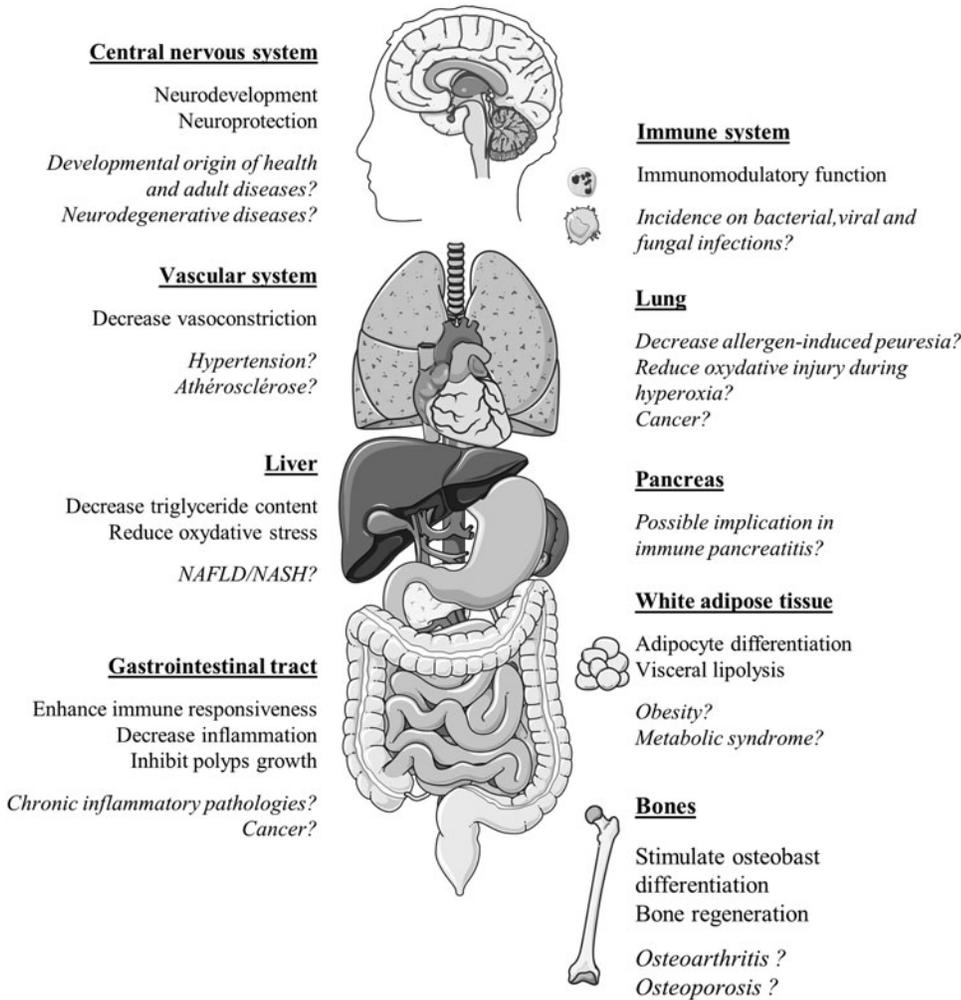


FIG. 3. Overview of global lactoferrin functions. General functions are shown for each organ. In italics are indicated pathologies where lactoferrin seems to play a role in the etiopathology and where it may constitute a therapeutic target. The Figure was made with Servier Medical Art.

the two lobes of hLf are highly important since their inactivation decreases iron affinity (223).

At physiological pH, Lf has a very high affinity for Fe^{3+} ($K_D \sim 10^{-22} M$) (7), but it is also able to retain iron even at pH 3.5. Kinetic studies conducted on hLf suggest that protonation of carbonate ion and amino acid (tyrosine and histidine) ligands at low levels is responsible for destabilization of the iron-binding site leading to domain opening and iron release (8). Nevertheless, further evidence is required to further elucidate the mechanisms by which iron is bound and released *in vivo* although these processes might implicate structural changes induced by binding to receptors, endocytosis/endosomal release, or Lf degradation. In addition to Fe^{3+} , Lf may bind to other ions such as Zn^{2+} , Cu^{2+} , Mn^{2+} , and Ce^{4+} but all with lower affinity than Fe^{3+} (9, 163). bLf can also bind to Ca^{2+} and this binding seems to implicate sialic acid present on the glycosylation sites (171).

The involvement of Lf in digestive iron absorption gained in credibility after the discovery of the intelectin receptor in the intestinal brush border membrane of rhesus monkeys (38). As Lf is strongly secreted in milk, numerous studies have investigated its role in iron supplementation and absorption in unweaned children. Currently, available studies on the role of Lf in iron absorption by the gastrointestinal tract are contradictory and need clarification. The use of a

milk based-formula supplemented with bLf induced an increase in serum ferritin levels compared with unsupplemented formula, suggesting that Lf may be involved in iron absorption (28). Likewise, the plasmatic iron of calves treated with iron sulfate and Lf is higher than in calves treated with iron sulfate alone, but only when Lf is fully iron saturated (94). A study involving young rhesus monkeys, whose digestive physiology is similar to that of young humans, has shown that a milk based-formula supplemented with hLf or bLf induces iron absorption similar to that obtained with the ferrous sulfate-supplemented formula (37). In contrast, other data using ^{59}Fe suggest that bLf is ineffective for iron absorption in rats and human newborns (47). Some reports have even shown a negative role of Lf in iron absorption. Davidsson *et al.* have found that the removal of Lf from human milk before feeding infants resulted in increased iron absorption (39). The same contradictions also appear in adults since healthy young women fed with a meal supplemented with radiolabeled ^{59}Fe -Lf or ferrous sulfate have the same iron absorption (110), whereas oral administration of bLf increases the number of red blood cells, hemoglobin, total serum iron, and serum ferritin in pregnant and nonpregnant women (155, 156).

Until now, the issue of Lf implication in iron absorption has been not resolved, but recent observations suggest that its importance is limited. This idea has emerged because of the

incompatibility of some findings with Lf as an active transporter. First, animal studies have failed to prove a direct role of Lf in dietary iron absorption. For example, Lf knockout mice do not exhibit reduced intestinal iron uptake, and mice that overexpress Lf do not have increased hemoglobin levels in their suckling offspring (67, 221). Second, healthy full-term birth weight infants are born with sufficient stores of iron to cover their needs during the first 4–6 months of life (41). Therefore, their needs in iron are limited compared to the high Lf level observed in human milk where only 5% of human milk Lf is iron saturated (117). Altogether, these data suggest additional roles of milk Lf to explain why human milk contains such a high fraction of iron-free Lf. Free iron may generate reactive oxygen species (ROS) that can damage the gastrointestinal tract through the Fenton reaction (30). The role of milk Lf may be to chelate iron to hold it in a stable nonreactive form and mitigate the local ROS production (30). These arguments are strengthened by the fact that Lf is able to chelate free iron even in acidic pH as observed in the stomach (127). In addition, microorganisms need free iron to grow. Therefore, by limiting the quantity of iron available for the need of microorganisms, milk Lf may participate in the installation of infant microbiota and prevent pathogen infection as discussed next.

Host defense, immunomodulatory, and anti-inflammatory functions

The focus of the role of Lf in immunity started with the discovery of specific granules containing Lf in granulocytes and when the absence of Lf in this granule led to recurrent infections (21). Since then, numerous observations confirmed the immunomodulatory functions of Lf (Fig. 3). Lf-deficient mice exhibited a stimulus-dependent defect in the oxidative burst response of neutrophils, which represents a necessary process for antibacterial defense (222). In addition, Lf-deficient mice, presenting the highest level of bacterial infections, displayed rapid bacterial clearance along with decreased host proinflammatory mediators following intravenous administration of hLf (213). Growing evidence indicates that the protein can not only protect against microbes but it also has the potential to directly immunomodulate host responses by regulating both innate and adaptive immunity (2,6,31).

By virtue of its antimicrobial properties, Lf is considered to be the body's first line of defense. It is secreted in most body fluids where it acts as a barrier against microbial infections. Some authors also consider Lf as an acute-phase protein of innate immunity (84). Indeed, Lf is released from granulocytes or epithelial cells at inflammatory sites. At this level, its function not only limits the proliferation of microbes but it also inhibits OxS induced by inflammation (102). Necrotic tissue releases free iron that may generate free radicals and lipid peroxidation, thereby amplifying inflammation (19). Lf released by granulocyte chelates this iron and is thus thought to limit OxS in patients with chronic hepatitis (90). Lf can also modulate immunity by blocking the binding of LPS to its receptors such as the soluble or membrane CD14 receptor and LPS binding serum protein. This disturbs the formation of the LPS-CD14 complex and results in an attenuation of the Toll-like receptor 4 signaling pathway at the surface of phagocytes and epithelial cells (99). However, Lf can modulate inflammation induced by LPS *via* other path-

ways. It has been demonstrated that hLf blocks OxS induced by LPS by competing with L-selectin, an independent LPS receptor in granulocytes (13).

Nevertheless, the role of Lf does not end there. Lf participates in the migration and activation of innate immune cells such as granulocytes. While this role is not fully understood, Lf seems to limit inflammation by inhibiting granulocyte migration (20). In pigs, Lf decreases the recruitment of eosinophils to the duodenum through the intestinal Lf receptor (33). Neutrophil mobility is also regulated by modulating the expression of metalloproteinase protein such as MMP matrix metalloproteinase-1 through the activation of activator protein-1 (150). Concomitantly, Lf increases the phagocytic activities of innate immune cells. However, the phagocytic activity of Lf seems to implicate other actors that are not fully identified (182). Simultaneously, Lf may also act on endothelial cells to inhibit inflammation. Lf slows down the recruitment of leukocytes mainly by decreasing the expression of adhesion molecules such as ICAM-1, E-selectin, and chemokines such as IL-8 by endothelial cells (14, 231). At this level, Lf seems to bind directly to the ICAM-1 promoter and thus inhibits the binding of nuclear factor- κ B (NF- κ B), thereby leading to the repression of ICAM-1 expression in endothelial cells during inflammation (87). In addition, a similar mechanism has been observed in monocytic THP-1, which leads to a decrease in NF- κ B binding to tumor necrosis factor- α promoter by Lf (69).

Regulation of apoptosis mechanisms and chemopreventive effects in targeting carcinogenesis

Studies using both *in vitro* and *in vivo* models have reported that human and bLf and their derived peptides can have beneficial effects in cancer treatment (63). The mechanism of Lf action is not fully understood but different pathways are highlighted. Lf may have an immunomodulatory function in case of infection. Therefore, it is not surprising that Lf may participate in the tumor growth inhibition by modulating immune functions. Oral administration of bLf was reported to exhibit the antitumor activity through the production of interferon- γ and IL-18 by immune cells in mice (93).

In addition, Lf may inhibit angiogenesis induced by tumorigenesis. Oral administration of bLf in transgenic mice overexpressing the human *VEGF-A165* gene, a model of pulmonary tumors, suppressed the formation of tumors by decreasing the expression of transgenes and inflammation (208). The mechanism of the antiangiogenic effect of Lf is not clear, but may implicate either the use of a specific Lf receptor or the inhibition of the action of some angiogenic proteins. For example, bovine Lf has been shown to form a complex with heparin-like structure on the surface of endothelial cells involved in the binding of *VEGF-165* and basic fibroblast growth factor to their respective receptors, thereby preventing receptor-stimulated angiogenesis (116). In addition, Lf has the potential to activate apoptosis in cancer cells. Indeed, diet supplemented with bLf decreased carcinogenesis by enhancing the expression of fatty acid synthase, caspase-3, and caspase-8, thereby leading to DNA fragmentation in rat colon mucosa (56). Likewise, the injection of recombinant adenovirus containing hLf in mice bearing EMT6 breast

cancer decreased the expression of Bcl-2 and increased Bax and caspase-3 expression, resulting in cellular apoptosis and tumor size reduction (220). bLf displays similar effects against breast cancer cells by inhibiting expression of surviving, an inhibitor of apoptosis proteins (62).

These results are highlighted by recent studies that have shown that iron-saturated bLf increased the chemotherapeutic effects of tamoxifen in the treatment of breast cancer in mice (193). In addition, the use of oral hLf in the treatment of the nonsmall-cell lung cancer showed encouraging results in a phase II clinical trial (42, 158). Finally, bLf has beneficial effects by blocking the growth of polyps that may lead to colon cancer (91). Altogether, these results suggest that the use of Lf could be beneficial for both the prevention and treatment of some cancers. Finally, Lf may also be used for targeted antitumoral gene therapy. Indeed, some cancer cells overexpress Lf receptors making Lf an excellent candidate for development of cancer-specific drug carrier coated with Lf that may be administered intravenously (107).

Lf and Oxidative Stress

Lf exhibits a functional role in the host's first line defense, thereby contributing to physiological responses at both the cellular and organ level (25, 32). This glycoprotein downregulates OxS at the molecular level, which facilitates the control of excessive inflammatory responses (15, 17, 92). Very often, the antioxidant action of Lf has been attributed to its iron sequestration capacity. However, using a UV irradiation-H₂O₂ system and the Fenton reaction, investigators show Lf direct OH[•] and O₂ scavenging potential along with protection against DNA double-strand breaks independent of its iron binding capacity (24, 149). In addition, the effectiveness of Lf to inhibit the production of thiobarbituric acid-reactive substances *via* 9-mer peptide within its core sequence—quite different from its iron binding sites—has been noted in an iron/ascorbate-induced liposomal phospholipid peroxidation system (218). Overall, these interesting findings indicate (i) protective features of Lf against lipid peroxidation that influences vital metabolic pathways through inactivation or modification of functional proteins and (ii) the possible interaction of LF molecules *via* a specific structure with oligonucleotides avoiding 8-OHdG formation and providing DNA protection from direct oxidative damage (71).

In addition to ROS quenching and DNA protection, there is an additional Lf mechanism of action that was revealed while investigating oxidative burst of neutrophils (159). Indeed, the antioxidant capability of Lf may counteract oxidative burst of neutrophils, which contributes to the pathogenesis of the septic shock (115, 219, 234). Lf molecules released from neutrophils during infection inactivate LPS, thereby preventing their binding to L-selectin on neutrophils and subsequent production of ROS by neutrophils, which limits the damage of tissues caused by excess production of oxygen radicals (14, 123).

Available data also suggest that Lf can alleviate OxS states by targeting mitochondria to preserve/regain their function. For example, in response to Lf, protective effects appear to result from either the preservation of mitochondrial Ca²⁺ homeostasis or the reduction of OxS-mediated damage in neurons, which preserves cellular bioenergetics (172). Moreover, it is reported that Lf protects against OxS-induced mitochondrial dysfunction and DNA damage, both in cell

culture and within an animal model of endotoxemia (113). Besides, deferoxamine, an iron chelator, provided only marginal protection of mitochondria, suggesting that Lf guards against oxidative insults at the cellular level *via* a more mitochondrial complex mechanism than simple iron sequestration. Interestingly, transcriptomal network analysis showed regulated changes at RNA levels during cellular response to Lf exposure (113). Various groups stressed the induction of signalling pathways or direct activation of nuclear DNA by Lf as a transcription factor was observed as well (53, 101). In particular, Lf seems to lessen oxidized base (purine) lesions (206) that are eliminated from the DNA by the base excision repair pathway, initiated by 8-oxoguanine DNA-glycosylase, a potentially protective cellular mechanism (72). Although the limited available data support the use of Lf in prevention and therapy to combat mitochondrial dysfunction and generation of ROS that culminates in ultrastructural mitochondrial abnormalities and signals for cell destruction within the affected tissue, there is a need to delineate to validate the findings and to delineate Lf mechanisms of action.

Lf And Metabolic Disorders

Insulin resistance and type 2 diabetes

Studies about the implication of Lf in glucose metabolism alteration started with the discovery of a negative correlation between circulating Lf concentrations and fasting glucose concentrations (134, 135) and a positive correlation between circulating Lf levels and insulin sensitivity (135). The authors described these observations in patients with altered glucose metabolism. However, Lf levels in T2D subjects seem similar to those in insulin-sensitive subjects (214). These divergent observations can, in part, be explained by the intense pharmacological therapy received by diabetic patients, which may influence plasma Lf levels. In view of the lack of specific data, the function of plasma Lf variation during insulin resistance (IR) is not fully understood. It is possible that Lf has a direct impact on IR in peripheral organs. Indeed, Lf was shown to improve the insulin-signaling response in mature adipocytes through the increase of AKT serine 473 phosphorylation and through an improvement in the expression of glucose transport 4 and insulin receptor 1 (136, 137). Further studies are required to understand the role of Lf in other organs implicated in IR such as the muscle and liver. However, as Lf is known for its anti-inflammatory and antioxidant properties, we do not exclude the possibility that its variations may be induced by low-grade inflammation and/or by OxS (134, 135). Finally, Lf plays a role in intestinal glucose absorption, particularly in inflammatory conditions. Indeed, intestinal prostaglandin E₂ released during inflammation is known to inhibit the Na⁺-glucose cotransporter (SGLT1) in enterocytes. Lf treatment can restore glucose transport by increasing the SGLT1 activity (202). Nevertheless, other studies are necessary to fully understand the role of both endogenous and exogenous Lf in glucose absorption and its potential implication in IR and T2D. Interventional studies in human involving Lf are required to resolve this issue.

Obesity

Numerous data have shown that circulating Lf levels are negatively correlated with both body-mass index (BMI) and

waist-to-hip ratio in overweight people (134, 135, 137, 214). Compared with overweight people, obese populations exhibit low circulating Lf (137). Microarray analyses performed on whole blood samples of nondiabetic Latino youth reveal an increase in the expression of Lf in overweight/obese participants, which leads to an increase in circulating Lf (89). These data are discordant with what has been shown in adults. However, these authors have shown a positive correlation of circulating Lf and age in male subjects, which could explain the difference between young and adult subjects (89).

It is well established that plasmatic Lf mainly comes from polynuclear neutrophils, but recent evidence reveals that adipose tissue and, more particularly, mature adipocytes express and secrete Lf. The expression of Lf in both subcutaneous and visceral adipose tissues is negatively correlated with both fat mass and BMI (Fig. 3) (137). Zhong *et al.* reported a decrease in Lf secretion during preadipocyte differentiation into mature adipocytes, while other studies have shown increased Lf cellular levels (137, 236). Altogether, these data suggest that circulating Lf levels may at least, in part, be regulated by metabolic and environmental stimuli (137, 236). The role of Lf in adipose tissue remains to be elucidated and various studies seem to show a dual mechanism of action. First, Lf seems to have antiadipogenic effects on 3T3-L1 preadipocytes, decreasing adipogenic gene expression that leads to a reduction in the development of lipid droplets (136). Treatment of mouse preadipocyte cell lines by Lf suppresses their adipogenic differentiation by reducing the expression of adipogenic transcription factors such as peroxisome proliferator-activated receptor-gamma and CCAAT/enhancer-binding protein-alpha (c/EBP α) (226). Conversely, the use of metformin as an antiadipogenic factor leads to a significant decrease in Lf gene expression during adipocyte differentiation (137). Lf increases the expression of two lipogenic genes, that is, fatty acid synthase and acetyl-CoA carboxylase in human primary adipocytes (133). Lf also enhances the expression of adipogenic genes in visceral and subcutaneous fat cells through an insulin-sensitizing effect (133). Finally, Lf knockdown results in a lower expression of adipogenic (adiponectin, acetyl-CoA carboxylase α , stearoyl-CoA desaturase-1) and insulin-related genes (glucose transport 4 and insulin receptor 1), but it is increased in inflammatory cytokines of differentiated adipocytes (132). Interestingly, these authors have shown that iron chelation has similar effects to Lf knockdown on adipocytes, suggesting that endogenous Lf participates in human adipocyte differentiation, possibly by modulating adipocyte iron homeostasis (132). Further studies will be necessary to clarify the function of Lf in adipose tissue.

Dietary LF consumption may represent a promising agent for the control of fat accumulation. Indeed, oral administration of Lf during caloric restriction in mice improved weight loss and induced a strong decrease in the weight of fat pad and adipocyte size (164). bLf administration decreased the size of mesenteric fat without modulating body weight in mice (138). Finally, in humans, an 8-week administration of enteric-coated Lf decreased total adiposity and specifically visceral fat accumulation (153). Although this phenomenon is not fully understood, it seems that exogenous Lf is rapidly detected in mesenteric adipose tissue in rats (152). These authors have also demonstrated that Lf and Lf treated with digestive enzymes (pepsin and trypsin) reduce lipid accumulation in preadipocytes possibly through a reduction in the

gene expression of c/EBP δ , c/EBP α , and peroxisome proliferator-activated receptor gamma (152). However, prospective and mechanistic analyses are still necessary to decipher the lipolytic role of dietary Lf and its potential implication in support of the treatment of obesity. In addition, the link among iron levels, Lf status, and obesity requires additional studies. This is particularly important since it has become evident that iron deficiency and obesity are molecularly linked and mutually affect each other (142). The role of Lf remains unknown in the two sides of iron–obesity relationship: (i) the mechanism leading to impaired iron balance with excess adipose tissue and (ii) the pathway of iron participation in obesity-related pathogenesis.

Potential implication in dyslipidemia and cardiovascular diseases

Circulating hLf has been shown to be associated with a decrease in fasting TG and an increase in high-density lipoprotein (HDL)-cholesterol and LDL-cholesterol (134). In morbidly obese subjects, there is no association among plasmatic Lf, TG, HDL-cholesterol, and free fatty acid levels (51). However, circulating Lf is inversely associated with free fatty acid levels after a fat overload (51). Although the mechanisms implicated in these correlations are not fully understood, experimental data seem to indicate that Lf displays beneficial effects on plasma lipid levels.

In mice fed standard diets, bLf reduced plasma and hepatic cholesterol and TG concentrations, with an increase in plasma HDL-cholesterol (200). However, a 4-week treatment with bLf failed to modulate plasma lipid parameters in ICR mice although it decreased hepatic TG content (138). The liver may develop metabolic impairments related to obesity, T2D, and nonalcoholic fatty liver disease. Recent studies have shown that Lf may have a protective effect on the liver. Using a murine model of liver injury, Li *et al.* demonstrated that oral bLf administration reversed weight gain, IR, plasma, and hepatic cholesterol, as well as TG profiles and the expression of proinflammatory cytokines induced by the administration of a high-fructose diet (104). Moreover, hepatic lesions induced by dimethylnitrosamine in rats were reduced by Lf administration, which decreased collagen production and activation of stellate cells (209). The exact mechanisms of the protection of the liver by Lf are not fully understood, but these studies suggest that Lf can limit OxS and inflammation during liver impairment (104, 209).

Despite the beneficial effects of Lf on lipid metabolism, its impact on the incidence of cardiovascular diseases is not clear. Circulating Lf levels are positively associated with coronary artery stenosis and the risk for fatal ischemic heart disease (214). In fact, a change in lifestyle by reducing dietary fat intake and increasing physical fitness leads to beneficial effects on the vascular system and induces a decrease in Lf transcription in leukocytes (46). However, experimental data suggest that Lf may also have a protective role in atherosclerosis etiology. Lf strongly inhibits cholesteryl ester accumulation from LDL by impairing its binding to macrophages and vascular smooth muscle cells (83, 109). Finally, Lf-derived peptide may have a potent antihypertensive function notably by blocking the angiotensin AT₁ receptor and by inhibiting the renin–angiotensin and the endothelin system (49, 59).

Impact on the intestinal microbiota

So far, numerous data have demonstrated that Lf exhibits antimicrobial effects on a wide spectrum of bacteria, fungi, viruses, and also parasites. For information about the microbial activity of Lf, the reader can consult the comprehensive review of Jensen and Hancock (80). In this part, we will focus on the Lf impact of intestinal microbiota because it is recognized as an important contributor to metabolism regulation and may take part in the development of metabolic pathologies (52, 75). Therefore, it is important to better understand the impact of endogenous and exogenous Lf on intestinal microbiota composition. A potential mechanism for Lf influence on metabolism may implicate its ability to change the body's microbiome composition. Lf has been shown to decrease the growth of some bacteria such as *E. coli* and *Salmonella spp.* and to promote the development of the *Bifidobacteria* strain (124, 148, 194). Thus, Lf may play a role in the regulation of metabolism through modifications in microbial composition. However, for the moment, this hypothesis testing has been largely neglected.

Clinical Interventions with Lf

In view of the numerous Lf functions described previously, it is not surprising that some studies have tried to show the safety of Lf and its potential beneficial health implications. Recently, a vast review of the various clinical interventions that use Lf, particularly in children, has been published (146). To date, 23 clinical trials using Lf have been recorded on ClinicalTrials.gov and numerous interventional studies have been published (Table 3). Most of these studies exploit the antimicrobial properties of Lf for prevention against nosocomial infection and some against different symptoms and pathologies, including psoriasis, acne, sepsis, diarrhea, colds, and anemia, as well as bacterial and viral infections (Table 3). These studies show very few or no adverse effects following Lf supplementation (146). In addition, bLf supplementation seems to be a promising strategy to reduce both the incidence of the first episode of late-onset sepsis and necrotizing enterocolitis in very low-birth-weight neonates (119). With regard to HIV infections, studies have shown that antiretroviral therapy supplemented by Lf does not lead to different viral loads, but leads to a higher CD4⁺ cell count and to an immune modulation response that could be useful (238). Interventional studies using a recombinant form of hLf named taLf have been conducted on certain patients with advanced nonsmall-cell lung cancer. Although mechanisms are not fully understood, oral supplementation of taLf seems to increase the overall survival in these patients, particularly in those with one or two prior lines of failed systemic anticancer therapy (42, 158). However, these effects have not been replicated in patients for whom two or more anticancer treatments failed (166). Thus, most clinical trials using Lf concern both anticancer and antimicrobial therapies. However, given the recent findings, it will not be surprising to see new clinical trials in the future that study the effect of Lf and derived peptides on stroke, hypertension, and metabolic disorders (210).

Preclinical Interventions with Lf

Given the considerable variety of biological functions of Lf, it is very surprising to note the extremely limited availability of intervention studies with Lf in cardiometabolic and

cardiovascular diseases. Only one report focused on visceral fat in a human clinical intervention (153). In a double-blind, placebo-controlled design, enteric-coated Lf (300 mg/d for 8 weeks) improved visceral fat-type obesity, an underlying cause of the metabolic syndrome (MetS), while decreasing body weight, BMI, and hip circumference without the need for any lifestyle change in Japanese men and women. The paucity of information regarding the role of Lf in cardiometabolic disorders led us to summarize the preclinical work in animals as shown in Tables 4 and 5. Using Lf whole-molecule or Lf-derived peptides, different groups showed antihypertensive effects *via* vasopeptidase (angiotensin I-converting enzyme/endothelin-converting enzyme) inhibition and anti-vasoconstrictor impact. Others were able to highlight their beneficial effects on the MetS components, including body weight control, IR lowering, dyslipidemia reduction, and hepatic lipid steatosis decline, as well as OxS and inflammation downregulation. Although evident advantages of Lf as a natural therapeutic agent against cardiometabolic disorders were stressed out in animal investigations, this role should clearly be extended in future clinical trials especially if one wants to designate Lf as a clinical tool in humans for diagnostic, therapeutic, and follow-up needs. Another issue is that the aforementioned studies identified several MetS molecular targets for Lf-derived peptides (reinforcing the great value of Lf as an effective source of multifunctional functions); however, clinical trials are necessary to demonstrate their separate and synergetic effects on MetS components.

Summary and Future Disorders

Lf research has achieved advances that have expanded the value of this protein in health promotion. However, it is important to continue the research efforts to elucidate its mechanisms of action. Three particular fields remain to be explored more deeply.

Lf synthesis, regulation, and mechanisms of action

What are the sources of circulating Lf? What is the importance of Lf isoforms and Lf-derived peptides? What mechanisms regulate endogenous Lf expression? What is the implication of epigenetic regulation, such as micro-RNA, histone acetylation, and DNA phosphorylation, on Lf synthesis? In addition, it is important to develop cellular and animal models to better understand the role of the Lf receptor in Lf action. These models may also lead to a description of intracellular signaling implicated in Lf action. Similarly, studies about the potential transcriptional activity of nucleus Lf are necessary.

Lf and physiopathology

Can Lf be an early marker of disease development? Data have also shown that Lf may be regulated in some physiopathological conditions and contribute to the development of pathologies. For inflammatory bowel diseases, leukocytes that infiltrate the intestinal mucosa release Lf, which may therefore be used as a marker of inflammation in the stools of patients (227). Whether Lf serves as a marker of detection or progression of other pathologies needs to be studied. For example, airway fluids from cystic fibrosis patients contain proteases that degrade Lf and seem to contribute to bacterial

TABLE 3. LIST OF THE DIFFERENT INTERVENTIONAL STUDY TRIALS USING LACTOFERRIN IN HUMAN

Subjects	Intervention	Results	References
22 subjects with mild to moderate plaque psoriasis	4-week trial with oral bovine Lf 100 mg + topical application of 10% or 20% of bovine Lf	Improvement in elevation, redness, and scaling. Oral Lf treatment did not exert any improvement	(176)
743 very-low birth weight neonates	From birth until day 30 of life, oral administration of bovine Lf (100 mg/day) with/without probiotic	↓ necrotizing enterocolitis incidence in groups receiving Lf	(119)
60 women at the 16th week of singleton gestation	Vaginal administration of 300 mg of Lf administered 4 or 12 h prior amniocentesis	Lf may exert a protective role complications by ↓ amniotic IL-6 level	(215)
126 participants with frequent upper respiratory tract symptoms and infections	Oral daily administration of 600 mg of Lf/IgF during 90 days	↓ number of cold reported ↓ cold-associated symptoms	(217)
71 tube-fed bedridden patients	Administration of an enteral formula containing Lf for 12 weeks	φ in immunological and nutritional variables ↓ incidence of fever in a subgroup of patients with low protein intake	(201)
227 patients with <i>Helicobacter pylori</i> infection	Administration of sequential therapy ± probiotic and/or Lf over a period of 2 years	Increase of compliance with the use of probiotic as an adjuvant to therapy, but addition of Lf to probiotic did not bring about any further improvements	(112)
163 pregnant women with iron deficiency or anemia	Oral administration of 100 mg of 20% iron-saturated bovine Lf, two times a day until delivery	Improvement of hematological parameters (red blood cell number hemoglobin, total serum iron, and serum ferritin)	(157)
30 patients with enteral feeding	subcohort of women with preterm delivery threat was treated with intravaginal Lf	↓ serum IL-6 level, for women treated with intravaginal Lf, diminution of uterine contraction and cervico-vaginal level of IL-6 and prostaglandin F _{2α}	(96)
472 neonates	Administration of 600 ml/day of human recombinant Lf-5 mg/ml) by gastrostomy tube during 8 weeks	↓ prevalence of diarrhea	(120)
20 subjects with periodontal disease	Administration of 100 mg/day of bovine Lf ± Lactobacillus rhamnosus GG	Decrease of incidence of invasive fungal infection	(78)
36 subjects with acne vulgaris	Oral administration of liposomal bovine Lf (180 mg/day) for 4 weeks	Decrease of total lesion count, sebum content, skin surface lipid	(88)
26 subjects with abdominal obesity (BMI >25 kg/m ² and visceral fat area >100 cm ²)	Daily ingestion of fermented milk with 200 mg of Lf for 12 weeks	Reduction of body weight, BMI, hip circumference, visceral fat area. No effect on blood biochemical or lipid parameters	(153)
148 anemic patients with advanced cancer undergoing chemotherapy	Oral administration of enteric-coated Lf (300 mg/day) for 8 weeks	Similar efficacy for oral Lf or iron administration for the treatment of anemia	(114)
104 participants with adenomatous colorectal polyps	Oral administration of two tables of Lf (200 mg/day) or intravenous iron administration. All patients received recombinant human erythropoietin (30,000 IU) once weekly for 12 weeks	High dose of Lf delay polyp growth	(91)
305 patients with severe sepsis	Oral administration of bovine Lf (1.5 or 3.0 g/day) for 12 months	No reduction of mortality	(216)
190 neonates with low birth weight	Oral Lf α 1.5 g for 28 days three times a day	Potential effect of Lf but need confirmation in a larger trial	(147)
555 children	Oral administration of 200 mg/kg per day of bovine Lf for 4 weeks	No decrease in diarrhea incidence but prevalence and severity were lowered	(145)
260 infants ages 4–6 months old	Oral administration of 0.5 g bovine Lf twice a day for 6 months	Lf-fortified milk increases iron absorption and total body iron content	(86)
	Oral administration of Lf-fortified milk formula (38 mg/100 g milk) for 3 month		

↓, down regulation.

TABLE 4. PRECLINICAL STUDIES EVALUATING THE IMPACT OF LACTOFERRIN ON THE METABOLIC SYNDROME COMPONENTS

Animal species (Males)	Lf			Outcomes	References
	Dose	Administration	Treatment length		
Rats with LPL-induced inflammation and hypotension	Bovine Lf (10 mg/kg)	G.G.	18 h	↓ Inflammation (TNF- α , IL-6) ↓ LPS-induced organ damage	(43)
SHR	Lf-derived peptide (10 mg/kg) (Lfcin B ₂₀₋₂₅)	G.G.	1–24 h	↓ Hypertension <i>via</i> inhibition of ACE ↓ ACE-dependent vasoconstriction	(173)
Rats with dexamethasone-induced hypertension	Bovine Lf (30–300 mg/kg)	G.G.	2 weeks	↓ Hypertension (prevention and reversal)	(174)
Rats	Bovine Lf (38.4–1280 nmol/kg)	I.V.	Acute administration	↓ Hypertension <i>via</i> activation of eNOS and endothelium-dependent vasodilatation	(70)
SHR	Lf peptides (10 mg/kg)	G.G.	1–24 h	↓ Hypertension with ACE-inhibitory potency	(59)
SHR	Lf peptides (200 mg/kg)	G.G.	1–24 h	↓ Hypertension <i>via</i> inhibition of ACE and ECE	(50)
SHR and Kyoto rat (WKY)	Lf peptides (1 pmol–1 nmol/ml/kg)	I.V.	0–120 min	↓ Hypertension <i>via</i> ACE inhibition	(100)
Mice	Bovine Lf (50–200 μ g/ml)	I.V.	90 min	↑ Postprandial lipemia by reducing chylomicron remnant from the liver	(76)
Mice model of inflammation	Bovine Lf (2.5–10 mg/body)	I.P.	24 h	↓ Inflammation (IL-1 β , TNF- α) ↓ Oxidative stress (myeloperoxidase) ↓ NF- κ B	(105)
ICR mice	Bovine Lf (100 mg)	G.G.	4 weeks	↓ Plasma TG ↓ Hepatic lipid accumulation	(138)
ICR mice	Bovine Lf (10 ng/kg diet)	Within diet	4 weeks	↓ Plasma TG ↓ Plasma nonesterified fatty acids ↓ Liver TG; ↓ Lymphatic TG	(200)
Holstein calves (injected with LPS)	Bovine Lf (1–3 g/day)	Oral	10 days	↓ Inflammation (IL-1 β , TNF- α , IL-6) ↓ TG; ↓ Fatty acids ↓ VLDL; ↓ HDL	(95)
Mice	Bovine Lf (190 ml/kg)	Oral	50 days	↓ Body weight; ↓ Obesity; ↓ Adipose tissue (visceral, abdominal) ↓ Plasma glucose	(164)

G.G., gastric gavage; HF, high fructose; MetS, metabolic syndrome; LPS, lipopolysaccharide; SHR, spontaneously hypertensive rats; TG, triglycerides; ACE, angiotensin-converting enzyme; ECE, endothelin-converting enzyme, I.P., intra peritoneal; ↓, down regulation; ↑, up regulation.

TABLE 5. LACTOFERRIN-DERIVED PEPTIDES WITH POTENT BIOLOGICAL EFFECTS

Peptides	Amino acid sequence	Concentration	Biological effects	References
LfCinB ₂₀₋₂₅	RRWQWR	20 μ M	↓ Vasoconstriction ↓ Hypertension	(118, 173)
LfCinB ₂₂₋₂₃	WQ	20 μ M	↓ Hypertension	(118, 173)
Fraction ₂₆₆₋₂₇₀	LIWKL	100 μ g/ml	↓ Vasoconstriction ↓ Hypertension	(118, 173)
Fraction ₁₃₃₋₁₃₆	RPYL	100 μ g/ml	↓ Vasoconstriction ↓ Hypertension	(118, 173)
Fraction ₂₃₂₋₂₃₈	LNNSRAP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₇₀₋₇₆	DPYKLRP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₇₁₋₇₆	PYKLRP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₇₂₋₇₆	YKLRP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₇₃₋₇₆	KLRP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₇₄₋₇₆	LRP	100 μ g/ml	↓ Hypertension	(118, 173)
Fraction ₁₃₂₋₁₃₄ 427-429 130-134	GILRP	100 μ g/ml	↓ Hypertension ↓ ECE activity and ECE-dependent vasoconstriction	(50, 118, 173)
PACE132D	RKWHFW	20 μ M	↓ ACE-dependent angiotensin-I contraction	(27)
PACE134D	RKWLFW	20 μ M	↓ ACE-dependent angiotensin-I contraction	(27)
P37D	RKKPFW	20 μ M	↓ ACE-dependent angiotensin-I contraction	(27)
hLfcin ₁₋₁₁	GRRRRSVQWCA	50-100 μ M	↓ Microbial activity	(184)
Lfcin ₁₇₋₃₀	FKCRRWQWRMCKLG	12.5-100 μ M 10 μ g/ml	↓ Microbial activity ↓ Myeloperoxidase and oxidative stress in macrophages	(184, 211)
hLf ₁₋₂₃	GRRRRSVQWCAVSQP EATKCFQW	250-500 μ M	↓ Viral activity	(54)
Lfcin ₄₋₁₄	RSVQWCAVSQP		↓ Cell proliferation ↓ DNA damage by UV light	(55)
hLACFR-Ia ₃₈₋₆₇	ITCCYPPTSVNRHTRK YWCRQGARGGCITL	1 μ M	↑ Bifidobacterial growth stimulatory activity	(106)
hPIGR-Ib ₁₀₁₋₁₂₂	SQDDSGRYKCGLGIN SRGLSFD	1 μ M		
Lf ₃₃	GRRRRSVQWCAVSQP EATKCFQWQRNMR KVRGP	0.001-2.5 μ M	Endotoxin neutralization	(235)
bLf ₆	Arg-Arg-Trp-Gln-Trp-Arg	1-20 μ M	Acting as an siRNA-delivering cell-penetrating peptide	(48)
Lf tryptophan- containing dipeptides	Val-Trp and Trp-Val	0.25 mg/ml	Xanthine oxidase inhibition	(144)

infections (170). For the moment, there are few studies that have investigated the association between mutations of the Lf gene and predisposition to diseases such as diarrhea and viral infections. It would also be interesting to study whether the presence of mutation in the Lf gene may predispose toward the development of pathologies.

Nutraceutical properties

Since Lf ingestion increases through the consumption of milk and derivatives, it is important to better understand the impact of this protein on health. First, we need to decipher the mechanisms implicated in Lf digestion and absorption by the gastrointestinal tract. Can this absorption be modulated and, if so, by what mechanisms? Can we artificially improve Lf absorption through the use of a galenic form? What is the

importance of Lf-derived peptide in health? It is necessary to answer these questions to develop serious nutrigenomic studies concerning the role of Lf in gene expression in particular as it implicates oxidative defense and inflammation. Finally, additional data are necessary to improve our comprehension of the role of Lf in metabolism, in particular in adipose tissues, lipolysis, and hepatic and intestinal lipid metabolism. In-depth studies are also needed on Lf's ability to modulate intestinal microbiota.

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Abbreviations Used

AA = amino acids
ACE = angiotensin-converting enzyme
bLf = bovine lactoferrin
BMI = body-mass index
bp = base pair
c/EBP α = CCAAT/enhancer-binding protein
alpha
ECE = endothelin-converting enzyme
Fe³⁺ = ferric iron
G.G. = gastric gavage
GAPDH = glyceraldehyde-3-phosphate
dehydrogenase
HDL = high-density lipoprotein
HF = high fructose
hLf = human lactoferrin

IL = interleukin
IR = insulin resistance
LDL = low-density lipoprotein
Lf = lactoferrin
Lfcin = lactoferricin
Lf-DP = lactoferrin-derived peptides
LPS = lipopolysaccharide
LRP = lipoprotein receptor-related protein
MetS = metabolic syndrome
NF- κ B = nuclear factor- κ B
OxS = oxidative stress
ROS = reactive oxygen species
SGLT1 = Na⁺-glucose cotransporter
SHR = spontaneously hypertensive rats
T2D = type 2 diabetes
TG = triglyceride