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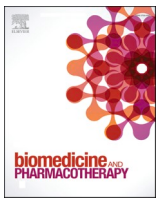

Review

Controversial role of lactoferrin in cancer: A narrative review

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ABSTRACT

Lactoferrin (Lf) is a positively charged iron-binding glycoprotein that has piqued the scientific community's interest due to its pleiotropic behavior, exhibiting a wide range of biological activities, including antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, and anticancer effects. This narrative review explores the current understanding of Lf's role in cancer, focusing on the endogenously expressed human full-length and ΔLf isoforms, and the effects of treatment with exogenous human and bovine Lf. We evaluated and compared the mechanisms by which Lf influences tumorigenesis and cancer progression, focusing on its impact on key processes such as cell growth, apoptosis, angiogenesis, cell migration, and invasiveness. Notably, the mechanisms of action of human and bovine Lf show some divergences, presumably due to slight structural differences that may lead to opposing effects. Insights from this comparison may help identify new Lf variants with enhanced anticancer activity. Indeed, both human and bovine Lf showed substantial anticancer activity, prompting researchers to investigate their potential utility in cancer prevention and progression. However, some studies have indicated that high levels of human Lf expression may be linked to increased carcinogenesis and metastasis, reflecting its intricate and context-dependent role in cancer. Thus, we emphasize the need for a deeper comprehension of Lf biology and its regulation to enhance understanding of Lf's role in cancer and identify more targeted therapeutic strategies, encouraging further research in this area.

1. Introduction

Lactoferrin (Lf), or lactotransferrin, is a multifunctional cationic glycoprotein and a member of the transferrin superfamily [1–3]. Lactoferrin is highly conserved among mammals. Human lactoferrin shares approximately 70 % amino acid sequence identity and a similar tertiary structure with other species, such as bovine, porcine, and murine [4]. The structure of Lf consists of two homologous globular domains, namely the N and C lobes, connected by an alpha helix, which share about 40 % sequence identity [5–7]. Each lobe contains a metal-binding site coordinated by four highly conserved amino acid residues [8,9]. Indeed, Lf is best known as an iron-binding protein, with a high capability to bind and transfer iron ions in a wide range of pH values, including strongly acidic [10,11]. This confers on Lf a key role in regulating iron homeostasis [12,13]. The levels of iron saturation may vary; indeed, Lf can exist as an iron-free "apo" form, in which the iron-binding sites are empty, and a highly iron-saturated "holo" form, in which the iron is coordinated by the two lobes of Lf. The binding of iron to Lf results in a conformational shift, where the protein undergoes a transition from an open, flexible structure in its apo form to a more closed, compact conformation in its holo form [14]. Interestingly, these conformational differences influence Lf stability and some functional features. In addition to iron, Lf is also capable of binding other metals, including Al^{3+}, Cu^{2+}, Mn^{3+}, and Zn^{2+}, which also contribute to affect Lf's structure, stability, and function [15]. Moreover, its cationic nature (primarily derived from the first 30 residues of the N lobe) and extensive glycosylation allow the protein to bind various biological molecules, such as DNA, lipopolysaccharide (LPS), and heparin [16]. Collectively, these properties highlight the versatility of Lf and its broad biological roles beyond iron sequestration, accounting for the wide range of biological functions associated with the protein, including antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, prebiotic, and anticancer activities [17–21].

The purpose of this work was to provide a narrative review of the role of Lf in cancer. We structured the review around two distinct but closely related topics. First, we examined the mechanisms of action underlying the antitumoral effects resulting from the endogenous expression of hLf and ΔLf isoforms, while also discussing some contrasting findings that

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associate high levels of hLf expression with the promotion of carcinogenesis and metastasis. Second, we investigated the anticancer effects of exogenous treatment with hLf and bLF, their putative role in cancer prevention and progression, and the mechanisms of action underlying these processes, considering the most relevant results obtained from *in vitro* and animal studies, as well as some clinical trials. Furthermore, we explored the potential factors that could account for some divergences in the anticancer effects exerted by hLf and bLf. We emphasized the importance of a more comprehensive understanding of these aspects to improve our comprehension of the complex role of Lf in cancer and the development of novel anticancer therapeutic strategies.

2. Role of lactoferrin in cancer

Diverse studies demonstrated the anticancer activity exerted by both exogenous bovine Lf (bLf) and human Lf (hLf), showing their role in inhibiting the growth and invasiveness of cancer cells by affecting various processes implicated in tumorigenesis and cancer progression, including cell growth, cell cycle progression, apoptosis, cell adhesion, and angiogenesis [22]. In humans, two main variants of lactoferrin (Lf) have been identified. These are the full-length secreted isoform, which is also present in other species, including bovine, and is found in diverse biological fluids (e.g., colostrum and milk, tears, saliva, sperm, and blood plasma), as well as in the secondary granules of neutrophils [23,24]. The second variant is the truncated isoform, such as delta-lactoferrin (ΔLf), which is localized intracellularly and has not been found in bovine or other species [25].

Interestingly, several studies reported that both the full-length secreted form and the cytosolic ΔLf are underexpressed in various types of cancer, including breast cancer [26,27]. In addition, stable or enhanced levels of protein expression have been shown to lead to a significant inhibition of cancer cell growth, suggesting their potential role as tumor suppressors [28–32]. On the other hand, other studies reported contrasting results and indicated hLf to be implicated in worsening the malignant phenotype of some cancer cells and promoting cell migration and metastasis [33]. Although the controversy remains heated, the strong involvement of both hLf and bLf in cancer is unambiguous. As for the mechanisms of action through which these proteins exert their activities on cancer, they are not yet fully understood. However, studies have shown that they can act differently depending on various factors, including cell localization, as seen in the case of the two hLf isoforms. Experimental evidence indicates that the full-length form triggers diverse signal transduction pathways by interacting with cellular receptors on target cells from different tissues [34,35], whereas the ΔLf isoform can undergo nuclear internalization [36], where it functions as a transcription factor [29].

2.1. Human lactoferrin: the full-length and delta-lactoferrin isoforms, a brief overview

Because of post-transcriptional and post-translational modifications and the alternative usage of start codons, hLf can exist in multiple variants [37,38]. Among these, the two main isoforms are the full-length secreted form (80 kDa) and the truncated intracellular form ΔLf (73 kDa), lacking the leader sequence and the first 26 amino acids (Fig. 1) [25]. Although ΔLf is deprived of a substantial part of the N-terminal region, which contains two clusters of positively charged residues and accounts for the significant antimicrobial activity displayed by the full-length protein, the truncated isoform maintains its alkaline features (ΔLf pI 8.19; full-length Lf pI 9) and related properties, including the capability of binding DNA and other anionic cell compounds [39]; however, the absence of the leader sequence determines its intracellular localization, and this could be one of the majors functional determinants and discriminants between the two isoforms, also in terms of the different post-translational modification pathways to which they are subjected, since *in silico* studies have not postulated any relevant structural differences [40].

The hLf gene is located on the third chromosome in the locus 3p21.31 [37] and consists of 17 exons [25]. The transcription of the two hLf isoforms is directed by the differential usage of two alternative promoters, namely P1 and P2. P1 is located upstream of exon 1 and its activation leads to the transcription of the full-length form of hLf. Conversely, P2 is located within the first intron and its activation leads to the transcription of the truncated ΔLf [27,42]. The mature mRNA of ΔLf contains exons from 1b to 17, with an untranslated region encompassing exon 1b and part of exon 2, while the mature transcript of the

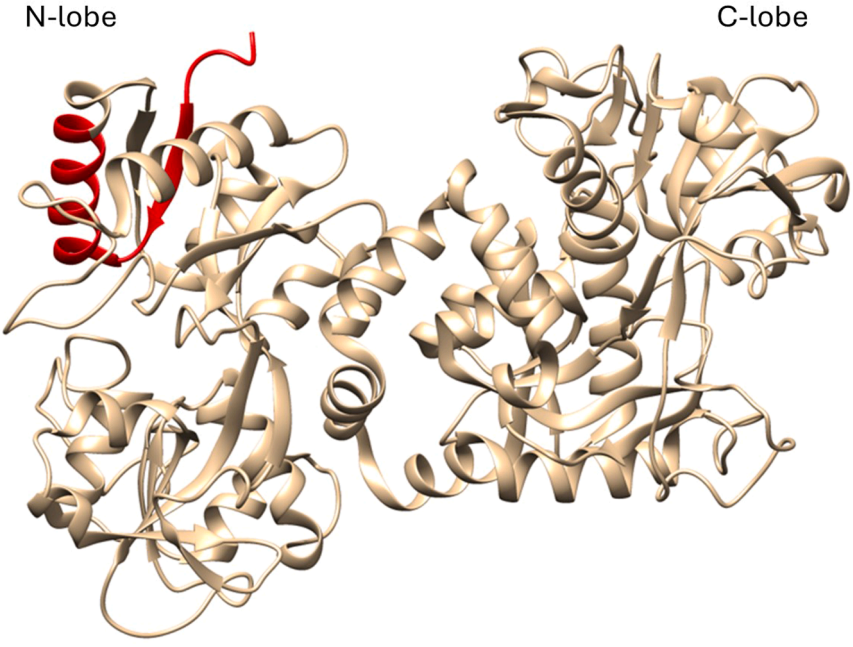

Fig. 1. Structure of hLf (PDB ID: 1B0L) [41]. The region of the protein highlighted in red represents the amino acid residues that are missing in the truncated ΔLf isoform. The figure was created using the UCSF Chimera package.

full-length Lf contains exons from 1 to 17 but lacks exon 1b, which is removed through alternative splicing (Fig. 2) [25].

The regulation of the hLf gene is a complex and multifaceted process, influenced by tissue- and cell-specific factors. Its expression can either be constitutive or regulated, depending on the cell types and tissues [43]. The presence of diverse constitutive and inducible P1 and P2 upstream regulatory elements, including estrogen response elements (ERE), Ets, Sp1, Sp3, C/EBP, NF-kB, and IKLF binding elements, accounts for the tissue- and time-specific differential patterns of hLf and/or ΔLf expression that have been reported [36]. Various molecular stimuli, including oxidative and inflammatory signals, steroid hormones, retinoic acid, LPS, dsRNA, and growth factors, have been shown to modulate the differential trans-activation of the two isoforms by triggering various signaling pathways [25]. For example, inflammatory stimuli like LPS primarily activate P1, inducing the transcription of the full-length isoform through pathways involving NF-kB and C/EBP transcription factors. Similarly, estrogen and retinoic acid regulate P1-driven transcription in a tissue-dependent manner.

In contrast, the P2 promoter, which governs ΔLf transcription, is strongly activated by Ets family transcription factors and exhibits minimal or no response to LPS, emphasizing a distinct regulatory mechanism that allows ΔLf to be expressed even in non-inflammatory conditions [27,42]. Interestingly, the expression of both isoforms can occur simultaneously in the same cell type, though at varying levels, reflecting their differential functional roles and the intricate regulatory dynamics of the hLf gene [27,44].

Although substantial progress has been made in understanding the regulation of hLf gene expression, key aspects remain unclear. In particular, the molecular switches that govern the balance between constitutive and inducible expression, as well as their modulation by environmental or cellular stimuli, warrant further investigation. Additionally, the specific pathways linking promoter activation to downstream functions remain only partially elucidated, suggesting an avenue for future research into the physiological and pathological roles of hLf regulation.

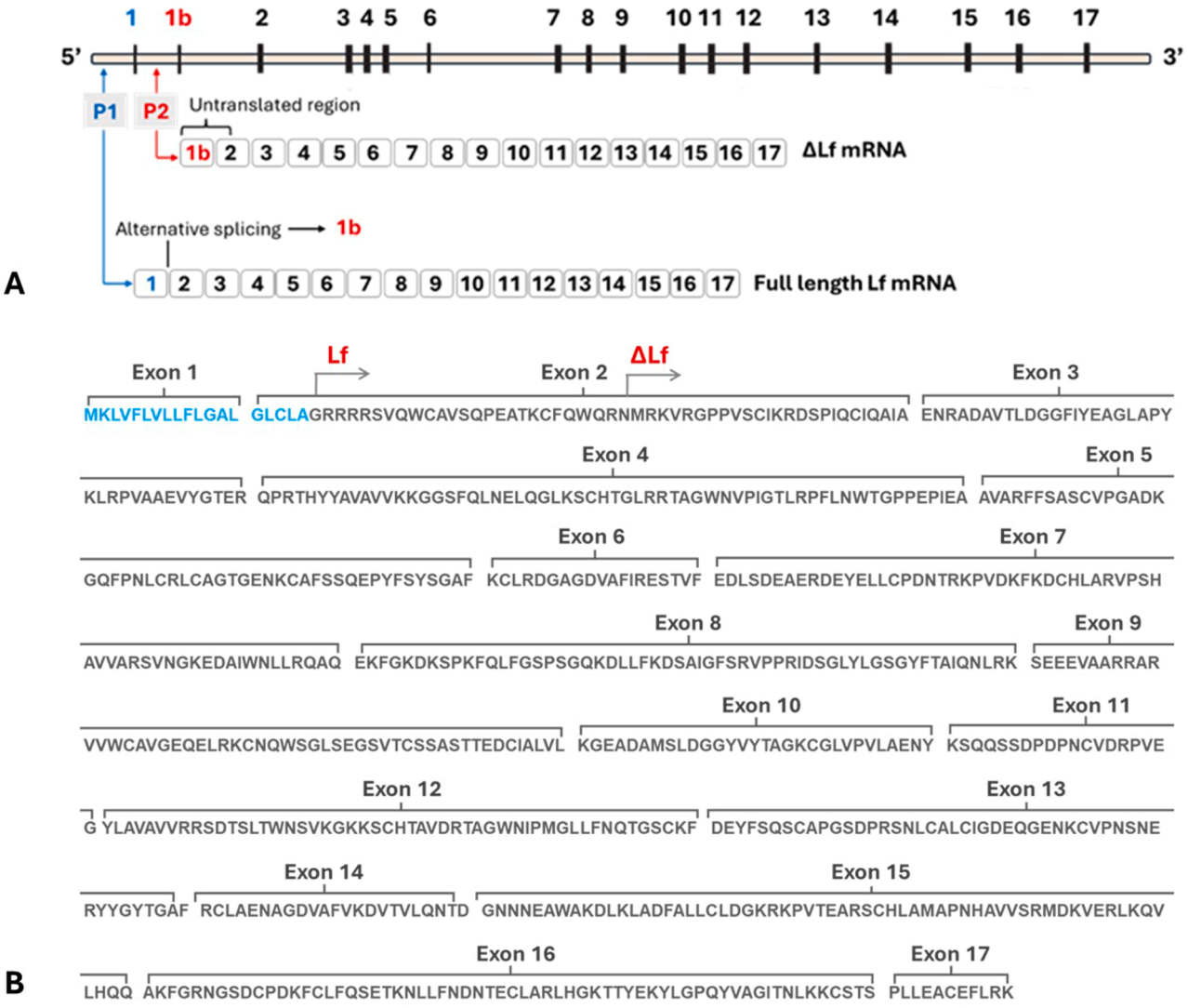

Fig. 2. A) Schematic representation of the hLf gene and the full-length hLf and ΔLf isoform transcripts. Differential activation of two alternative promoters, P1 (located upstream of exon 1) and P2 (located within the first intron), leads to the transcription of the full-length isoform and the truncated ΔLf, respectively. The mature mRNA of ΔLf contains exons from 1b to 17, with an untranslated region encompassing exon 1b and part of exon 2. The mature transcript of the full-length Lf contains exons from 1 to 17 but lacks exon 1b, which is removed through alternative splicing. B) Sequence of full-length hLf and ΔLf isoforms. The amino acid sequences encoded by each exon of the hLf gene have been reported. Highlighted in blue is the signal peptide, which directs the full-length isoform through the secretory pathway (not present in the mature form) [25,37].

2.2. Mechanisms of action underlying the antitumoral activity of human full-length and ΔLf isoforms

Alterations in the hLf gene, including mutations, chromosomal aberrations, and changes in the methylation levels and patterns, resulting in the downregulation or absence of both full-length and ΔLf, have been observed in various types of cancer [27,45,46]. For example, in nasopharyngeal carcinoma (NPC), studies have shown that Lf expression is absent or significantly downregulated in 76 % of primary NPC tissues (25 out of 33 cases). Interestingly, 63.6 % (21 out of 33) of the primary NPC samples exhibited hypermethylation at the Lf promoter. This methylation-induced silencing of Lf expression was further confirmed by the restoration of Lf transcript levels in NPC cell lines upon treatment with the demethylating agent 5-aza-2-deoxycytidine, which reversed the methylation mark and reactivated Lf expression [47]. Another study, conducted in prostate cancer cells, has found that CpG island near Lf's transcriptional start site is frequently and densely methylated. Analysis using methylation-sensitive techniques and bisulfite sequencing confirmed this hypermethylation, even in early stages like high-grade prostatic intraepithelial neoplasia. These findings suggest that Lf mRNA transcription is silenced through hypermethylation, reinforcing its role as a tumor suppressor gene in prostate cancer. Furthermore, the high frequency of Lf CpG island methylation across samples highlights its significance as a critical and conserved event in the initiation of prostate cancer [48]. Indeed, the involvement of lactoferrin isoforms in cancer has been suggested by results from several studies that confirmed the antiproliferative, antimetastatic, and pro-apoptotic activities of lactoferrin and identified the protein as a potential tumor suppressor [49,50]. Notably, the interplay between Lf isoforms and cancer pathways has gained significant attention due to their ability to influence various stages of cancer progression. In this context, separate mechanisms of action for the two isoforms have been proposed. Regarding the full-length isoform, the leader sequence at its N-terminal conveys the protein towards the secretory pathway. As a secreted protein, it acts mainly exogenously, and the interaction with cell receptors is crucial for its functions. Indeed, studies have demonstrated that the anticancer activity of the full-length hLf is associated with its ability to modulate various cell signaling pathways, including those involved in cell cycle progression (e.g., MAPK, AKT, NF-kB and TGFβ/Smad-2 pathways) and apoptosis (e.g., JNK pathway), as well as to the modulation of the signaling cascades involved in immunomodulation and immunostimulation [51]. A study performed on head and neck cancer cells demonstrated that treatment with hLf caused cell cycle arrest at the G0-G_1 checkpoint. The authors showed this was due to the Lf-mediated modulation of AKT phosphorylation, which affected the p27/cyclin E-dependent signaling pathway [52]. Results from another *in vitro* study showed that hLf exerts antiproliferative effects by inducing increased levels of p21 expression and modulating the expression and the phosphorylation state of the retinoblastoma protein (Rb) [53]. hLf has also been shown to inhibit tumorigenesis, cell growth, and invasion in nasopharyngeal carcinoma cells by inhibiting the expression of 3-phosphoinositide-dependent protein kinase 1 via the mitogen-activated protein kinase/c-Jun pathway, thus suppressing the AKT signaling pathway [54]. Other studies demonstrated the hLf-mediated inhibition of the AKT signaling pathway, which was reported to induce proapoptotic effects in SGC-7901 human stomach cancer cells [55]. A study performed on Jurkat leukemia T cells demonstrated that hLf induces apoptosis in a dose- and time-dependent manner by intersecting the JNK-associated Bcl-2 signaling pathway [56].

The extensive N-glycosylation to which this Lf isoform is subjected plays a pivotal role in these processes; hLf glycosylation sites embrace Asn138, Asn479, and Asn624. Indeed, besides protecting from proteolysis and reducing protein immunogenicity, glycosylation accounts for many of the full-length Lf functions, including its defensive role against pathogens (e.g., preventing epithelial cell adhesion), immunomodulatory and anticancer activities [57,58]. N-glycans are known to be involved in mediating cell recognition, cell adhesion, and cell receptor binding [16,59]. Interestingly, the glycosylation pattern may vary depending on the tissue where the protein is expressed. According to that, studies performed on bLf demonstrated that low Lf glycosylation levels strongly inhibit Lf-mediated cell signaling and the internalization of the protein by affecting the processes above. In addition, the same study has demonstrated the role of Lf glycosylation in positively modulating the capability of the protein to inhibit cancer cell growth and proliferation by improving surface adhesion, Lf internalization, and inhibition of the ERK/Akt pathway [60]. In addition to glycosylation, the two positively charged amino acid clusters at the N-terminal lobe also promote the interaction of Lf with cell receptors, which allows the binding to cell glycosaminoglycans. Results from studies performed on MDA-MB-231 breast cancer cells showed the importance of this interaction in modulating the Lf-mediated effects on cell proliferation and cancer progression [61]. Although the full-length Lf mainly acts exogenously, studies have demonstrated that the association of the protein with Lf cell receptors can lead to protein internalization in diverse types of cells [62–65]. *In vitro* studies on Caco-2 cells showed that the protein is internalized via clathrin-mediated endocytosis, and once internalized, it modulates cell proliferation by intersecting the ERK signaling pathway [66]. In addition, the GRRRR sequence at the N-terminal of the full-length Lf (residues 1–5) has been identified as a nuclear localization signal (NLS) region [67]. This, together with the capability of Lf to bind DNA [68], suggest that the full-length isoform may have an additional role as a transcription factor. However, strong evidence of its nuclear localization is lacking, and its involvement as a transcription factor has not been fully documented [36,69].

On the contrary, ΔLf maintains an intracellular localization and can enter the nucleus. Indeed, even if ΔLf lacks the GRRRR NLS region, another NLS (RRSDTSLTWNSVKGKK) is present at the C-terminal lobe (residues 417–432), which has been demonstrated to mediate its nuclear internalization [70]. Interestingly, ΔLf response elements have been found at the promoters of many genes, and studies have shown that ΔLf acts as a transcription factor targeting diverse genes, including those involved in S-phase cell cycle arrest and apoptosis [40,71]. A study by Mariller and colleagues [72] demonstrated that ΔLf binds to the S-phase kinase-associated protein (Skp1) gene promoter at two different sites (GGCACTGTAC-located at - 1067 bp, and TAGAAGTCAA- located at - 646 bp) enhancing its expression. Skp1 is a protein involved in the ubiquitination and degradation of diverse cell cycle regulators, and it has been suggested that ΔLf may influence cell cycle progression by regulating its expression [72]. Interestingly, the GGCACTGTAC ΔLf response element sequence is also responsible for ΔLf-mediated IL-1β transactivation [71]. Later studies identified other ΔLf target genes. In a proteome profiling study performed on transiently transfected HEK 293 and human cervical cancer HeLa cell lines, and in stably transfected MDA-MB-231 breast cancer cells the protein changes induced by ΔLf were assessed. Results reported that ΔLf transactivates transcription from the scavenger mRNA-decapping enzyme (DcpS) gene promoter. DcpS plays a crucial role in mRNA decay, and the authors suggested that its upregulation may have a strong role in affecting cell viability [73]. In another proteomic study, a SILAC-based proteomic profiling approach was used to evaluate the transcriptional effects of ΔLf in breast cancer cells, employing a stably transfected MDA-MB-231 cell line as a model. The results indicated that ΔLf enhances the expression of many genes involved in cell proliferation, apoptosis, ubiquitination, oxidative stress, and mRNA quality control, including Selenoprotein H, IIF2, and ubiquitin-conjugating enzyme E1 genes [29]. Indeed, studies have shown that the upregulation of ΔLf is associated with antitumoral effects. Among these, results from an *in vitro* study demonstrated that the overexpression of ΔLf modulated the proliferation of HEK293 cell lines by inducing cell cycle arrest in the G1/S phase [70]. Results from another *in vitro* study demonstrated that ΔLf expression induced apoptosis in HEK 293 and MCF7 cell lines through the intrinsic mitochondrial-death pathway. The authors showed that ΔLf interacts

with a ΔLfRE at the Fas and Bax promoters, leading to their transcriptional activation [74]. Based on these findings, targeting the transcriptional activity of ΔLf could be of great importance for a better understanding of its antitumoral activity. In this context, engaging data emerged from *in silico* studies, which identified various putative O-N-acetylglucosaminylation, SUMOylation, and phosphorylation sites, suggesting that these, like for other transcription factors, could be involved in regulating the transcriptional activity of the protein [40]. Moreover, some authors suggested that modulating ΔLf's transcriptional activity could be helpful to identify new potential therapeutic strategies [40,73]. Interestingly, a later study provided experimental evidence on the repressive action of SUMOylation in ΔLf's transcriptional activity and its influence on enhancing ΔLf stability; furthermore, the authors demonstrated that, on the contrary, acetylation leads to ΔLf transcriptional activation [75]. Notably, SUMOylation has emerged as a critical regulatory mechanism influencing cell proliferation, migration, and metastasis across various cancer types. In breast cancer, studies have shown that it interacts with other post-translational modifications, forming a complex crosstalk that governs the diverse stages of cancer progression [76].

2.3. Assumed pro-tumoral effects of human lactoferrin

By virtue of the antiproliferative and pro-apoptotic activity of endogenous full-length Lf and ΔLf, it is plausible to assume that the loss of lactoferrin or reduced levels of expression could provide a selective advantage for cancer cells. However, the outcomes of these findings remain controversial, and the role of Lf in cancer is not yet fully elucidated. Indeed, although many types of cancers have been associated with low expression levels of both full-length and ΔLf, and studies have provided evidence of the pro-tumorigenic and pro-metastatic effects of hLf deficiency [31], other studies—contrasting with *in vivo* research that demonstrated the anticancer effects mediated by hLf [77,78]—reported opposite results, showing that high levels of hLf worsen the malignant phenotype of cancer cells and promote cancer cells migration and metastasis [79–81]. Interestingly, in the study conducted by Hoedt and colleagues [29], the authors reported that, in parallel with the increase in the expression of genes involved in antiproliferative and proapoptotic mechanisms, high levels of hLf induced an increase in the expression of some genes whose products are known to their pro-metastatic effects or to be associated with malignant phenotypes, including cathepsin z, gamma-glutamyl hydrolase and heparanase [29]. In another study, treatment of the human breast cancer cell line MCF7 with Lf led to enhanced expression levels of ULK1, a protein involved in autophagy. Moreover, the study showed the role of Lf in inducing the autophagy initiation complex [82]. These results, together with other studies that demonstrated the involvement of Lf in modulating autophagy, further emphasized the ambiguous role of Lf in cancer [83,84]. Indeed, autophagy plays a dual role in cancer, suppressing tumor initiation and supporting uncontrolled cell growth in the later stages of the disease [85].

Another point of note is the role of hLf in some hormone-sensitive cancers, including breast cancer. The presence of two half-palindromic estrogen response elements (EREs; GGTCA), an imperfect ERE (GGTCAAGGCGATC), and the Steroid Factor 1 Response Element (SFRE) at the hLf gene promoter regions accounts for hLf estrogen's responsivity. Indeed, studies have shown that estrogen receptor α (ERα) binds to EREs and induces hLf gene expression [86,87]. However, studies have noted an inverse correlation between hLf levels and ERα [81,88,89]. Interestingly, an *in vitro* study performed on breast cancer cells showed that hLf may contribute to the emergence of the highly invasive triple-negative phenotype (TNBC) by mediating the down-regulation of estrogen receptor-α (ER-α), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). The study demonstrated that Lf induced the expression of endothelin-1 (ET-1) and suggested that the Lf-ET-1 axis is implicated in the occurrence of the TNBC phenotype and in favoring migration and invasiveness [81]. This can have important implications. First, by promoting the TNBC phenotype, hLf can impact the possibility of treating this cancer since these receptors are usually used as therapeutic targets [90,91]. Even more, a consequence of the downregulation of ERα is the increase in the expression and functionality of the estrogen-related receptor α (ERRα). In the absence of ERα, ERRα modulates in an estrogen-independent manner the expression of a specific subset of EREs containing genes, including its exclusive target genes and some of those usually under the control of ERα. ERRα is known to play a crucial role in modulating breast cancer cell signaling pathways [92], and studies have reported that this condition is associated with an increased risk of cancer recurrence, metastasis, worsened clinical outcomes, and poor prognosis [93–95]. However, results from a clinical retrospective study investigating Lf immunoexpression in a cohort of 65 TNBC cases showed a correlation between Lf expression and less aggressive disease [96], supporting similar findings from previous studies that associated tumors with high levels of Lf with a more favorable prognosis [97]. Additionally, results from an *in vitro* study conducted by Iglesias-Figueroa and colleagues [98] suggest that the effects of hLf may vary between normal and cancer cells and depend on the cancer cell phenotype. The authors aimed to evaluate the cytotoxic effects of human recombinant Lf (hrLF) on a panel of six human breast cancer cell lines, including triple-negative and normal breast cells, noting selective hrLf-mediated cytotoxicity between normal (i.e., MCF-10A) and cancerous cells, with the former being significantly less affected (CC_{50} 1279.48 μg/mL). Moreover, the study found that metastatic cell lines (i.e., MDA-MB-231) were more affected by the treatment (CC_{50} 109.46 μg/mL) than non-metastatic ones, being, however, non-metastatic triple-negative less affected (MDA-MB-468 and HCC70; CC_{50} values: 491.8 and 823.6 μg/mL, respectively) than the non-metastatic ER, PR and CR/NC3C1-positive cell lines (i.e., MCF-7; CC50 382.2 μg/mL) [98].

Another controversial area within the role of hLf in cancer concerns its involvement in angiogenesis, which is known to be crucial in tumor growth and metastasis [99]. Although one of the various mechanisms by which bLf exerts its antitumoral activity relies on its ability to inhibit angiogenesis [100–102], hLf has been found to have opposite effects, showing proangiogenic activity, as reported by the results of *in vitro* and *in vivo* studies. An *in vivo* study conducted on rats showed that the oral or subcutaneous administration of the apo form of hLf significantly enhanced VEGF-A-mediated angiogenesis and that, on the contrary, apo-bLf showed a significant inhibitory action on the process [103]. Another study carried out *in vitro* on endothelial cells (HUVECs) confirmed the proangiogenic effects of hLf, showing that it enhances VEGF-induced cell migration and proliferation by upregulating KDR/Flk-1 expression [104].

2.4. Anticancer activity of bovine lactoferrin

The structure of bLf is almost the same as hLf; indeed, although there are some differences in their sequences, the two proteins share 69 % homology (Fig. 3). bLf, similarly to hLf, before being further processed into its mature form of 689 amino acids, contains an N-terminal signal sequence of 19 amino acids that directs the protein toward the secretory pathway. However, unlike hLf, a truncated cytosolic isoform of bLf has not been identified.

Numerous *in vitro* and *in vivo* studies and some clinical trials have investigated the anticancer effects of the exogenous treatment with bLf [22].

An *in vitro* study investigating the antiproliferative and anti-metastatic effects of holo-bLf and apo-bLF in breast cancer cells demonstrated that both variants of bLf were effective in inducing apoptosis in MDA-MB-231 and MCF-7 cell lines through a mechanism involving the inhibition of the protein surviving. No cytotoxic effects were observed in normal MCF-10–2A cells [105]. Similarly, another *in vitro* study on oral squamous cell carcinoma cell lines revealed that bLf

Fig. 3. Sequence alignment between bLf and hLf obtained using the BLAST (Basic Local Alignment Search Tool) program. The amino acid sequence underlined in red refer to the signal peptide, which is not found in the mature proteins.

inhibits tumor growth by inducing apoptosis and suppressing proliferation. This effect was mediated through the induction of p53 expression and modulation of mTOR/S6K and JAK/STAT3 signaling pathways [106]. A study conducted on cervical cancer HeLa cell, aimed at investigating the regulatory effects of bLF on the proliferation, apoptosis, and autophagy, demonstrated that bLF significantly reduced proliferation in a dose- and time-dependent manner. Additionally, bLF induced apoptosis by upregulating proapoptotic proteins, including p53, Bax, and Cleaved-caspase-3, while downregulating the antiapoptotic protein Bcl-2. Furthermore, bLF treatment activated autophagy-related proteins such as LC3B-II and Beclin I and decreased the expression of the autophagosome transporter protein p62, suggesting that bLF induces autophagy in HeLa cells. Importantly, pretreatment with the autophagy inhibitor 3-MA reversed bLF-induced apoptosis, indicating that bLF induces apoptosis by activating autophagy in HeLa cells [107]. A study conducted on SGC-7901 human stomach cancer cell lines demonstrated that bLf inhibits the proliferation of cancer cells by inducing apoptosis through the activation of Akt and the modulation of the phosphorylation of its downstream proteins [55]. Another study showed that both apo-bLf and holo-bLf are internalized by DU-145 prostate cancer cells, leading to reduced cell viability, changes in cell morphology, cellular damage, and cell cycle arrest, thereby providing evidence for the antitumoral activity of bLf. The study further reported that the holo form of bLf demonstrated enhanced activity, presumably due to its higher stability relative to the apo form [108]. Notably, a separate study revealed that bLf-mediated cell death is highly selective for metastatic prostate cancer cells, with no effects on non-cancerous cells. The authors attributed this selectivity to the inhibitory activity that bLF exerts on the V-ATPase, which is highly abundant in the plasma membrane of various highly metastatic cancer cells [109].

bLf has been shown to play an important role in reducing cell migration and invasiveness of various cancer cell types. An *in vitro* study on human glioblastoma cell lines demonstrated that bLf hinders cell migration by influencing the epithelial-mesenchymal transition (EMT)-like process and inhibiting the IL-6/STAT3 axis [110]. A more recent study found that bLf inhibits oral squamous cancer cell invasion, which is induced by tPA, MMP-1, and MMP-3, through inactivation of AP-1 complexes [111]. Another *in vitro* study reported that bLf reduces migration and invasiveness in MDA-MB-231 breast cancer cells. Consistently, the holo-Lf form exhibited stronger effects compared to the apo form. Furthermore, the findings strongly suggest that bLf exerts its anti-metastatic effects on breast cancer cells by inhibiting the epithelial-mesenchymal transition process [112].

The anticancer effects of bLf have also been reported in various *in vivo* studies [113–115]. Among these, an interesting study by Iigo and colleagues [116] used murine models to assess the molecular pathways activated by bLf that could underlie its antitumoral effects. They noticed that the oral administration of bLf triggered the activation of several immunomodulatory pathways in the small intestine mucosa, such as the IL-18/Caspase 1/IFϒ or IFα/IL-7, depending on the mouse model. The authors suggested that the subsequent activation of the immune cells might be implicated in protecting against tumor development [116]. Another study conducted in diabetic BALB/c mice showed that bLf exerts a suppressive effect on the development of type 2 diabetes-induced colon cancer by regulating the PI3K/AKT/mTOR signaling pathway and inducing the phosphorylation of 5′-nucleotidase domain-containing 3 (NT5DC3) protein, a protein known for its role in promoting colon cancer in diabetic patients [117]. Other significant results came from an *in vivo* study focused on assessing the antimetastatic action of bLf in murine models inoculated with tumor cells. The study demonstrated that the subcutaneous administration of apo-bLf, in synergy with one of its peptide derivatives, lactoferricin-B, was effective in suppressing liver, spleen, and lung metastasis, as well as inhibiting tumor-induced angiogenesis and tumor growth. Additionally, different time points of action for apo-bLf and lactoferricin-B were reported, with lactoferricin-B being active only in the early phases after administration. The author suggested that these molecules exert different mechanisms of action. The activity of lactoferricin-B was associated with its ability to disrupt cell membrane functionality, while the activity of apo-Lf was correlated with its iron saturation levels. Indeed, treatment with holo-Lf was found to be ineffective in suppressing metastasis and tumor growth [118].

Altogether, these results suggest that Lf could be an up-and-coming candidate to cancer prevention and treatment. However, although encouraging results have emerged from some clinical trials, demonstrating Lf's capability to inhibit the growth and progression of certain intestinal non-malignant neoplastic formations [119,120], further studies are necessary to assess the effectiveness of exogenous administration of Lf in cancer treatment.

One of the major challenges to overcome is the efficacy of the Lf delivery to target sites, in terms of ensuring enhanced stability, solubility, bioavailability, and target specificity. Recent reviews have extensively covered the most advanced nanomaterial-based strategies for targeting cancer that could be efficiently applied to Lf delivery [121, 122].

3. A comparison of the anticancer activity of hLf and bLf

Based on these results, it appears clear that the effects of bLf in cancer overlap with those described for hLf, both influencing processes involved in cell growth, apoptosis, angiogenesis, cancer cell migration, and invasiveness. However, the mechanisms by which the two proteins exert these functions are, in some cases, dissimilar, intersecting distinct

signaling pathways and targeting different molecules, which can lead to different or even opposite effects. Nevertheless, compared to hLf, studies on the role of bLf in cancer are much more defined and less controversial; to our knowledge, only a very few studies have reported conflicting results. The reasons for these differences could be ascribed to various factors. In addition to considering the different effects that may arise from the administration of exogenous bLf and full-length hLf, or from the action of physiologically regulated endogenous hLf isoforms, the structural differences between these proteins may play a crucial role. Indeed, slight differences in their sequences lead to very similar but not identical structures and physicalchemical features. In this context, the presence of diverse N-glycosylation sites, in terms of number, position, and the type of glycans attached (hLf contains three potential glycosylation sites, Asn138, Asn479, and Asn624; while bLf five, Asn233, Asn281, Asn368, Asn476, and Asn545), should be considered [16]. Additionally, the differences between their N-terminal domain sequence could have a determinant role. The mature form of full-length hLf begins with the strongly basic sequence GRRRR, which is unique among species and may be an important factor in differentiating the molecular interactors of these molecules and/or their binding efficiency [123]. In contrast, bLf contains only one basic residue among first five amino acids of its sequence. These considerations could be extended to some of their peptide derivatives; indeed, human lactoferricin contains a greater number of positively charged amino acids compared to bovine lactoferricin [124]. The importance of this has been remarked by the results from studies conducted on bovine lactoferricin, which demonstrated that the presence of a net positive charge and the length of the peptide are crucial factors in determining the effects that these bLf peptide derivatives exert on cancer cells [125]. Moreover, an *in vitro* study conducted on diverse cancer cell lines aimed at investigating the anticancer effects of human lactoferricin and some of its variants provided interesting insights into the characteristic features that may lead to improved anticancer activity. The authors reported that some sequence variations in human lactoferricin, which lead to the formation of an alpha-helix structured loop, led to a significant (10-fold) increase in cancer cell cytotoxicity, compared to the native peptide, resulting in a stronger effect on selective induction of apoptosis in cancer cells by targeting the negatively charged membrane phosphatidylserine [126].

Another aspect concerns the levels of iron saturation, which have been found to play a role in influencing the anticancer effects of Lf. Studies showed that apo- and holo-Lf forms, mainly due to structural differences, can intersect different signaling pathways, suggesting differences in the binding to molecular targets [127]. Indeed, as reported in some of the previously discussed studies, apo-Lf is more effective than the holo-form [110]. However, which of the two Lf iron-saturated forms exerts greater activity could depend on the environmental and experimental context. Indeed, conversely, another study reported increased anticancer properties for the holo-Lf, which, compared to the apo form, was shown to have a much stronger effect in inducing ferroptosis in triple-negative MDA-MB-231 breast cancer cells and in sensitizing the tumor to radiotherapy [128]. However, these considerations apply to both hLf and bLf.

Altogether, these diverse features could influence and differentiate the binding of these proteins to cell receptors and molecular interactors, including transcription factors and gene promoters [4,129–131]. In this context a case in point comes from the previously mentioned study that demonstrated the antiapoptotic and pro-metastatic effects of hLf on TNBC cells. As reported in this study, one of the primary mechanisms by which hLf supports cell proliferation and invasiveness in TNBC cells is the upregulation of endothelin-1, a process driven by the direct binding of hLf to the ET-1 gene promoter. Specifically, three hLf binding sites were identified within the ET-1 promoter region [81]. By contrast, bLf exhibited proapoptotic and antimetastatic effects. Interestingly, no evidence of bLf binding to the ET-1 promoter has been reported [33,132].

Given the importance of these issues in providing new insights into the anticancer activity of Lf and advancing the search for molecular variants of Lf with enhanced anticancer properties, further studies focused on expanding our current knowledge on these aspects should be encouraged. In this regard, a recent study provided intriguing results by investigating the anticancer activity of various engineered variants of hLf, developed through a rational design approach. The study was conducted on different cancer cell lines, including gastric (AGS), breast (MCF7), and colorectal cancer (HCT116 and CT26) cells. The authors found that one of these variants, truncated at its N- and C-terminal lobes, exhibited up to 100-fold greater anticancer activity than the full-length hLf. Interestingly, the structural features of this variant were found to enhance the stability of the protein and result in a 3-fold reduction in its iron-binding capability when compared to the full-length Lf. Moreover, this engineered hLf variant was demonstrated to upregulate diverse pro-apoptotic proteins, such as those involved in the activation of the Bax/Bak signaling pathway, while downregulating proteins linked to angiogenesis, cell migration and invasiveness [133].

Despite these differences, an important common feature of the two proteins is their strong selectivity against cancer cells. Diverse studies have highlighted that both hLf and bLf exert contrasting effects on cell growth, cell migration, DNA synthesis, and inhibition of apoptosis, depending on whether they interact with normal or cancer cells [134–138]. This strengthens the potential of Lf as an effective molecule for supporting cancer prevention and countering progression. However, it is noteworthy that this selectivity may arise from differences in how Lf interacts with cancer cell surfaces compared to normal cells. Indeed, as with V-ATPase, some anionic components of cell membranes, such as sialic acid and phosphatidylserine, are overexpressed in many cancer cells [139,140] and, even in this case, the two proteins may present differences in terms of effectiveness.

4. Conclusions

Although several studies have reported strong anticancer activity for both hLf and bLf, suggesting their potential effectiveness in cancer prevention and treatment, the role of these proteins in cancer and the underlying mechanisms of action are still not completely understood. To complicate the picture, some contrasting results have emerged from studies on hLf, which have associated high levels of its expression with the promotion of cancer cell proliferation and invasiveness, as well as the worsening of the malignant phenotype of some cancer cells (i.e., TNBC); this highlights the complex biology of Lf. Indeed, besides the Lf destabilizing effects on cell-wall and membranes, and the effects related to its iron-sequestering capability, at the root of the Lf effects on tumorigenesis and cancer progression are additional factors such as: i) its capability to bind cell receptors (which in some cases leads to Lf internalization) and other molecular interactors ii) its capability to intersect diverse signaling pathways, which is the consequence of its molecular interactions; iii) its capability to bind DNA, thus targeting different genes and modulating their expression.

In this review, we first examined the role of the endogenous expression of hLf in cancer, focusing on the different mechanisms of action underlying the functions of the full-length protein and its truncated intracellular form, ΔLf. In this context, the complex regulation of the hLf gene stands out. This should be one of the first points to be closely investigated, given the importance that a deeper understanding of this could have, not only to better understand the mechanisms of action of the two hLf isoforms but also to improve knowledge of their role in tumorigenesis and cancer progression. Moreover, especially in the light of some controversial results that have emerged regarding the role of these proteins in cancer, having more data on their regulation could be essential to understand whether and in what cases their expression could be advantageous or potentially detrimental. This could open the possibility of developing more targeted and context-dependent therapeutic strategies.

We also reviewed the effects of exogenous treatment with hLf or bLf in cancer, and results from *in vitro* and *in vivo* studies have reported that

both proteins, as well as their peptide derivatives, exert strong anti-cancer activity, influencing processes involved in cell growth, apoptosis, angiogenesis, cancer cell migration, and invasiveness. However, the mechanisms by which the two proteins exert these functions are, in some cases, different, intersecting diverse signaling pathways and/or targeting different molecules, potentially leading to different or opposite effects, depending on the context. Thus, we evaluated the structural differences that could potentially underlie the functional divergences between hLf and bLf, as this can be undoubtedly useful for a better understanding of the mechanisms of action underlying their anticancer activity. In addition, this comparison may also provide important information that could lead to the rational design of engineered Lf variants with increased anti-cancer activity; thus, improving the research in this field could be of great importance in the search for new molecules that could find applications in cancer therapy.

Lastly, even though results from *in vitro* and *in vivo* studies rank Lf as a promising candidate for cancer treatment, results from clinical trials are still too lacunose to assess the effectiveness of exogenous treatment with Lf in preventing or controlling cancer progression in patients. Indeed, performing clinical trials in this field is currently hampered by several factors, including the complex structure of the protein, which limits the effective delivery of Lf to the target sites. In this context, advancements in nanomaterial-based drug delivery could help identify more effective Lf delivery strategies that combine high specificity to the target, greater stability, solubility, and bioavailability.

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CRediT authorship contribution statement

Valentina Gallo: Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Giovanni Antonini:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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