

# Role of lactoferrin and its receptors on biliary epithelium

Romina Mancinelli · Francesca Olivero · Guido Carpino · Diletta Overi ·  
Luigi Rosa · Maria Stefania Lepanto · Antimo Cutone · Antonio Franchitto ·  
Gianfranco Alpini · Paolo Onori · Piera Valenti · Eugenio Gaudio

Received: 2 January 2018 / Accepted: 13 March 2018 / Published online: 17 March 2018  
© Springer Science+Business Media, LLC, part of Springer Nature 2018

**Abstract** Human lactoferrin is an iron-binding glycoprotein present at high concentrations in breast milk and colostrum. It is produced by many exocrine glands and widely distributed in a variety of body fluids. This protein has antimicrobial, immunomodulatory, antioxidant, and anticancer properties. Two important hLf receptors have been identified: LDL receptor related protein (LRP1), a low specificity receptor, and intelectin-1 (ITLN1), a high specificity receptor. No data are present on the role of hLf on the biliary epithelium. Our aims have been to evaluate the expression of Lf and its receptors in human and

murine cholangiocytes and its effect on proliferation. Immunohistochemistry and immunofluorescence (IF) were conducted on human healthy and primary biliary cholangitis (PBC) liver samples as well as on liver samples obtained from normal and bile duct ligated (BDL) mice to evaluate the expression of Lf, LRP1 and ITLN1. Cell proliferation in vitro studies were performed on human cholangiocyte cell lines via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as well as IF to evaluate proliferating cell nuclear antigen (PCNA) expression. Our results show that mouse and human cholangiocytes express Lf, LRP1 and ITLN1, at higher extent in cholangiocytes from BDL and PBC samples. Furthermore, the in vitro addition of bovine Lf (bLf) has a proliferative effect on human cholangiocyte cell line. The results support a proliferative role of hLf on the biliary epithelium; this pro-proliferative effect of hLf and bLf on cholangiocytes could be particularly relevant in human cholangiopathies such as PBC, characterized by cholangiocyte death and ductopenia.

---

R. Mancinelli (✉) · F. Olivero · D. Overi ·  
A. Franchitto · P. Onori · E. Gaudio  
Department of Anatomical, Histological, Forensic and  
Orthopedic Sciences, Sapienza University of Rome,  
Rome, Italy  
e-mail: romina.mancinelli@uniroma1.it

L. Rosa · M. S. Lepanto · A. Cutone · P. Valenti  
Department of Public Health and Infectious Diseases,  
Sapienza University of Rome, Rome, Italy

G. Carpino  
Department of Movement, Human and Health Sciences,  
Sapienza University of Rome, Rome, Italy

G. Alpini  
Research, Central Texas Veterans Health Care System,  
Baylor Scott & White Digestive Disease Research Center,  
Baylor Scott & White, Department of Medical  
Physiology, Texas A&M University College of Medicine,  
Temple, TX 76504, USA

**Keywords** Biliary epithelium · Cholangiocytes ·  
Lactoferrin · Intelectin-1 · Primary biliary cholangitis

## Introduction

Human lactoferrin (hLf) is a multifunctional iron binding glycoprotein of 691 amino acids, produced by exocrine glands and neutrophils (Berlutti et al. 2005).

This glycoprotein belonging to the transferrin family has a highly conserved structure among human, bovine, mouse, and other species (Berlutti et al. 2011). Many studies have unraveled the numerous functions of hLf: host defense, antimicrobial and anti-inflammatory, iron homeostasis, cellular growth and differentiation, and anti-cancer activity (Frioni et al. 2014, Sessa et al. 2017, Zhang et al. 2014). In particular hLf, by its iron-chelating ability, influences bacterial growth, aggregation and biofilm formation (Berlutti et al. 2008, Cutone et al. 2014). Other than by chelating iron, hLf performs multiple biological activities depending on both target cell and receptor or by interacting with other molecules, such as lipopolysaccharide (LPS), glycosaminoglycans (GAGs) (Ward et al. 2005) and DNA (Albar et al. 2014). Lactoferrin receptors (LfR) have been shown to be differently expressed depending on tissue and cell specificity (Suzuki et al. 2005). Intelectin1 (ITLN1) is a lactoferrin receptor that was first discovered in the intestinal epithelium, responsible for hLf uptake from the digestive tract. The ITLN1 is a glycoprotein made up of three 40-kDa subunits cross-linked by disulfide bonds making up a 120-kDa homo-trimer of 295 amino acids and N-linked oligosaccharides (Akiyama et al. 2013). Furthermore, other than in the intestinal brush border, it is also present in Paneth and goblet cells (Wrackmeyer et al. 2006). Reverse transcriptase PCR studies have revealed that the mRNA for ITLN1 is expressed in various tissues, explaining the wide range of functions hypothesized for hLf, possibly via activation of cell signaling pathways. Another LfR is the LDL receptor related protein 1 (LRP1), which is a low specificity receptor for hLf, as it can bind multiple targets. LRP is a member of the LDL receptor gene family, to which it is structurally similar. It is a type I transmembrane receptor of 600 kDa, composed of five subunits (Shi et al. 2009). LRP1 is abundantly expressed in hepatocytes, neurons, smooth muscle cells, and fibroblasts (Suzuki et al. 2008). Other than binding hLf, LRP1 has a diverse array of functions: lipoprotein metabolism, proteinase metabolism, activation of lysosomal enzymes, cellular entry for viruses and toxins, as well as a possible role in neurotransmission (Herz and Strickland 2001).

The biliary tree is a ductal system of increasing diameter through which bile flows to the gallbladder and intestine after being produced by hepatocytes (Wei et al. 2015). The epithelial cells lining the biliary tree

are cholangiocytes. Small bile ducts are lined by small cuboidal cholangiocytes, while larger ones are lined by thicker columnar cholangiocytes (Wei et al. 2015). Small and large cholangiocytes differ in their dimensions, ultrastructure, functions and proliferative capabilities (Glaser et al. 2009). The morphological and functional heterogeneity of the intrahepatic biliary epithelium has been studied also in experimental animal models such as the bile duct ligated (BDL) rat or mouse, in which there is a selective proliferation of large but not small cholangiocytes (Mancinelli et al. 2013). The pathophysiology of small cholangiocytes is largely unknown (Glaser et al. 2009). Primary biliary cholangitis (PBC) is an auto-immune chronic cholestatic liver disease characterized by the destruction of small intrahepatic bile ducts, leading to fibrosis and potential cirrhosis. PBC is characterized histologically by a chronic, non-suppurative inflammation surrounding and destroying the interlobular and septal bile ducts, sparing the large intra- and extra-hepatic ducts (Carpino et al. 2015). Moreover, there is a relationship among inflammation, collagen deposition, ductopenia and liver damage progression: these features are used to define the “stage” of PBC according to the Ludwig classification (Ali et al. 2016): Stage I consists of portal inflammation with the typical “florid bile duct lesions”; Stage II consists of the extension of the inflammatory process to the periportal areas with loss of normal bile ducts and development of bile duct reduplication; Stage III is characterized by septal fibrosis or inflammatory “bridging” with progressive loss of bile ducts; Stage IV is frank cirrhosis with end-stage liver disease (Carpino et al. 2016).

With this background, our aims have been: i) to evaluate the expression of Lf and its receptors by cholangiocytes in vivo and in vitro both in human samples (normal subjects and PBC patients) and in mouse (normal and BDL), and ii) to evaluate the effects of Lf on biliary epithelium proliferation.

## Materials and methods

### Reagents

Reagents were purchased from DBA ITALIA srl (Milan, Italy), unless otherwise indicated. Primary antibodies for immunohistochemistry and immunofluorescence were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA), Abcam (Cambridge, UK), DAKO (Agilent technologies, CA, USA). In detail, we used: polyclonal anti-human Lactoferrin, (DAKO, F0395), monoclonal anti-mouse Lactoferrin, (Abcam, ab10110), monoclonal anti-human LRPI, (Santa Cruz, sc-57351) and polyclonal anti-human ITLN1, (Abcam, ab118232). Secondary antibodies (anti-mouse and anti-rabbit) with fluorophores (Alexa Fluor 488 and Alexa Fluor 594) for immunofluorescence were purchased from Invitrogen Thermo Fisher Scientific (Waltham, MA, USA). Real-time PCR primers and other reagents were purchased from SABiosciences (Frederick, MD, USA). The cell proliferation assay kit (MTT) to evaluate the cellular growth was purchased from Millipore (Massachusetts, USA).

#### Bovine lactoferrin (bLf)

Highly purified bLf was kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan). The purity and integrity of bLf was checked by SDS-PAGE and silver nitrate staining, while its concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The bLf iron saturation was about 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation, 1% solution). LPS contamination of bLf, estimated by Limulus Amebocyte assay (Pyrochrome kit, PBI International), was equal to  $0.5 \pm 0.06$  ng/mg of bLf. Before biological assays, bLf solution was sterilized by filtration using 0.2  $\mu$ m Millex HV at low protein retention (Millipore Corp., Bedford, Mass.). In all experiments, bLf was used at a non-cytotoxic concentration corresponding to 100  $\mu$ g/ml.

#### Animal models

Male C57/BI6 N mice (20–25 g) were purchased from Charles River (Wilmington, MA), kept in a temperature-controlled environment with 12:12-hour light–dark cycles and free access to water and standard chow. The studies were performed in 7 normal mice and in 15 mice that underwent ligation of the main bile duct (BDL) for 1 week before the sacrifice. All animal experiments were performed in accordance with a protocol approved by the Scott and White and Texas A&M HSC IACUC Committee. All animals were

used for the harvest of tissues and purification of liver cells.

#### Human samples

The study was carried out on liver biopsies from 15 postmenopausal females, 10 patients with diagnosis of PBC, while 5 liver biopsies from organ donors showing a normal histology. PBC specimens were staged according to Ludwig et al. (1978) as stage III and IV. All PBC patients and liver donors were negative for hepatitis B and C markers. The study protocol was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

#### Purification of cholangiocytes

Pure cholangiocytes (by GGT histochemistry) were isolated from mice and human biopsies by immune-affinity separation using a monoclonal antibody that recognizes a specific antigen expressed by all intra-hepatic cholangiocytes. Cell number and viability (greater than 97%) were assessed by standard Trypan blue exclusion. Then, cholangiocytes were seeded on slides to create smears and fixed in acetone for 10 min. These smears have been used for the immunofluorescence and MTT cell proliferation assay.

#### Immunohistochemistry (IHC)

Specimens were fixed in 10% buffered formalin, embedded in low-temperature-fusion paraffin and cut in sections of 3  $\mu$ m. For IHC, endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (3%). Sections were then incubated overnight at 4 °C with the specific primary antibodies. Samples were rinsed twice with PBS, incubated at room temperature with secondary biotinylated antibody (LSAB+ System-HRP, Dako) and then with Streptavidin-HRP (LSAB+ System-HRP, Dako). Diaminobenzidine (DAB, Dako) was used as substrate, and sections were counterstained with hematoxylin. Immunohistochemical observations were taken in a coded fashion by a light microscopy Leica Microsystems DM 4500, with a camera Jenoptik ProgRes C10 Plus (Jena, Germany) and analyzed with an image analysis system (Delta Sistemi, Rome, Italy). For all immunoreactions, negative controls (with

normal serum from the same species substituted for the primary antibody) were included.

### Immunofluorescence (IF)

For IF on hepatic specimens from mice and human, after deparaffinization, sections were hydrated in graded alcohol and rinsed in phosphate-buffered saline and 0.1% Tween20 (PBS-T) and then incubated with 10% normal blocking serum in PBS. After washing, slides were incubated overnight at 4 °C with the primary antibody diluted in PBS with 1.5% normal blocking serum. Samples were rinsed in PBS-T and incubated for 45 min at room temperature in a dark chamber with the specific fluorophore-conjugated secondary antibody diluted in PBS with 1.5% normal blocking serum. For double immunofluorescence, we incubated the primary antibodies at the same time if they were originated from different species or in two times if produced in the same type of animal. The samples were washed in buffer and mounted with UltraCruz mounting medium. Pictures were taken by DM4500B light microscopy (Leica, Wetzlar, Germany).

On cell culture or smears, fixed cells were incubated with the specific primary antibodies. Then, cells were washed and incubated for 1 h with labelled isotype-specific secondary antibodies (anti-mouse AlexaFluor-546, anti-goat AlexaFluor-546, anti-mouse Alexafluor-488, anti-rabbit Alexafluor-488, Invitrogen Thermo Fisher Scientific—Waltham, MA, USA) and counterstained with 4, 6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei. For all immunoreactions, negative controls (the primary antibody was replaced with pre-immune serum) were also included. Smears/Cultures were examined in a coded fashion by Leica Microsystems DM 4500 B Light and Fluorescence Microscopy (Wetzlar, Germany) equipped with a JenoptikProg-Res-C10 Plus Videocam (Jena, Germany). IF staining was also analyzed by Aperio Scanscope-FL System (Aperio Technologies, Inc, Oxford, UK) and processed by ImageScope.

### Real time PCR

To evaluate the transcript expression of lactoferrin and ITNL1 (high specificity receptor) in purified cholangiocytes from normal and BDL mice, we used the RT<sup>2</sup>

**Fig. 1** **a** Immunohistochemistry in liver sections. Cholangiocytes in normal and BDL mouse bile ducts are positive for lactoferrin. The protein is also present in normal human liver and in stage IV PBC liver samples. In PBC, Lf expression is markedly increased with respect to normal liver. **b** Expression of LRP1, the low specificity receptor, was weak in normal and BDL mouse bile ducts. In human samples, we found its expression to be strongly enhanced in stage IV PBC sample. **c** Expression of ITLN1, the highly-specific receptor, was present in normal and BDL mouse bile ducts. In normal human liver it was not so evident, while there was a markedly increased expression in bile ducts in stage IV PBC samples. Original magnification  $\times 40$

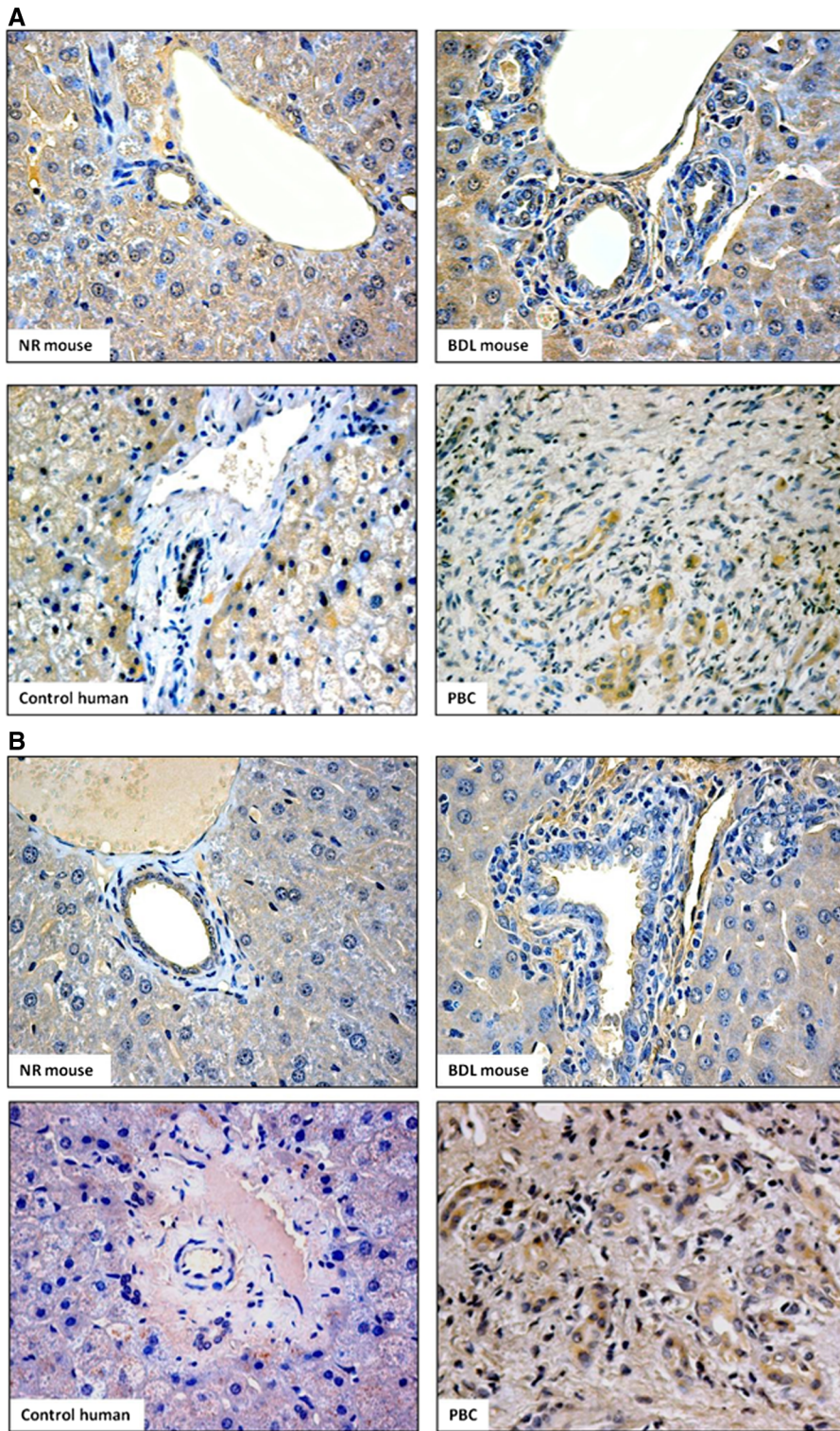
Real-Time assay from SABiosciences (Frederick, MD). A  $\Delta\Delta C_T$  (delta delta of the threshold cycle) analysis was performed using normal cholangiocytes as the control sample. The primers for lactoferrin and ITNL1 (purchased from SABiosciences) were designed according to the NCBI GenBank Accession. Data were expressed as relative mRNA levels  $\pm$  SE of Lactoferrin and Intelectin-1 to GAPDH ratio.

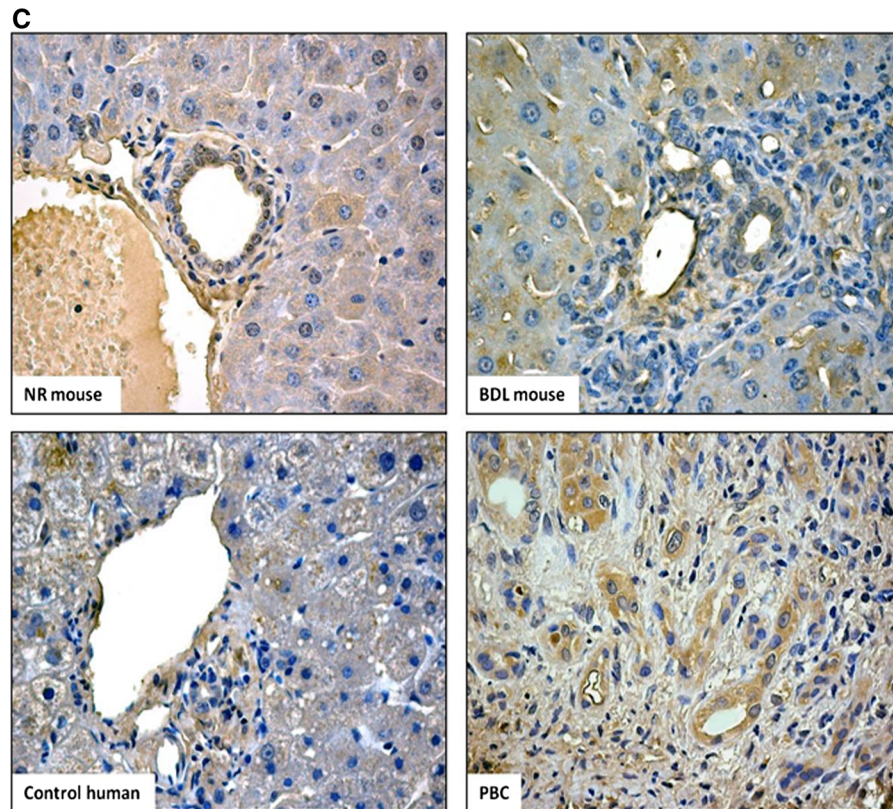
### Proliferation evaluation

A human immortalized non-malignant cholangiocyte cell line (H69, a gift of Prof. Gianfranco Alpini, Texas A&M University, Temple, TX, USA) were seeded into 96-well plates (10,000 per well) in a final volume of 200  $\mu$ l of growth medium and allowed to adhere to the plate overnight. Cells were stimulated with bovine lactoferrin (bLf) (100  $\mu$ g/ml) at 37 °C for 24 h before evaluation of cell growth through the Cell Proliferation Assay kit (Millipore). The formazan dye produced by viable cells can be quantified by a microplate reader (LT-4000 labtech) by measuring the absorbance of the dye solution at 450 nm.

### Statistical analysis

Data are presented as arithmetic mean  $\pm$  standard deviation (SD). Student's *t* test was used to determine differences between groups from normally or not normally distributed data, respectively. A *P* value of  $< 0.05$  was considered statistically significant. Statistical analyses were performed using the SPSS statistical software.





**Fig. 1** continued

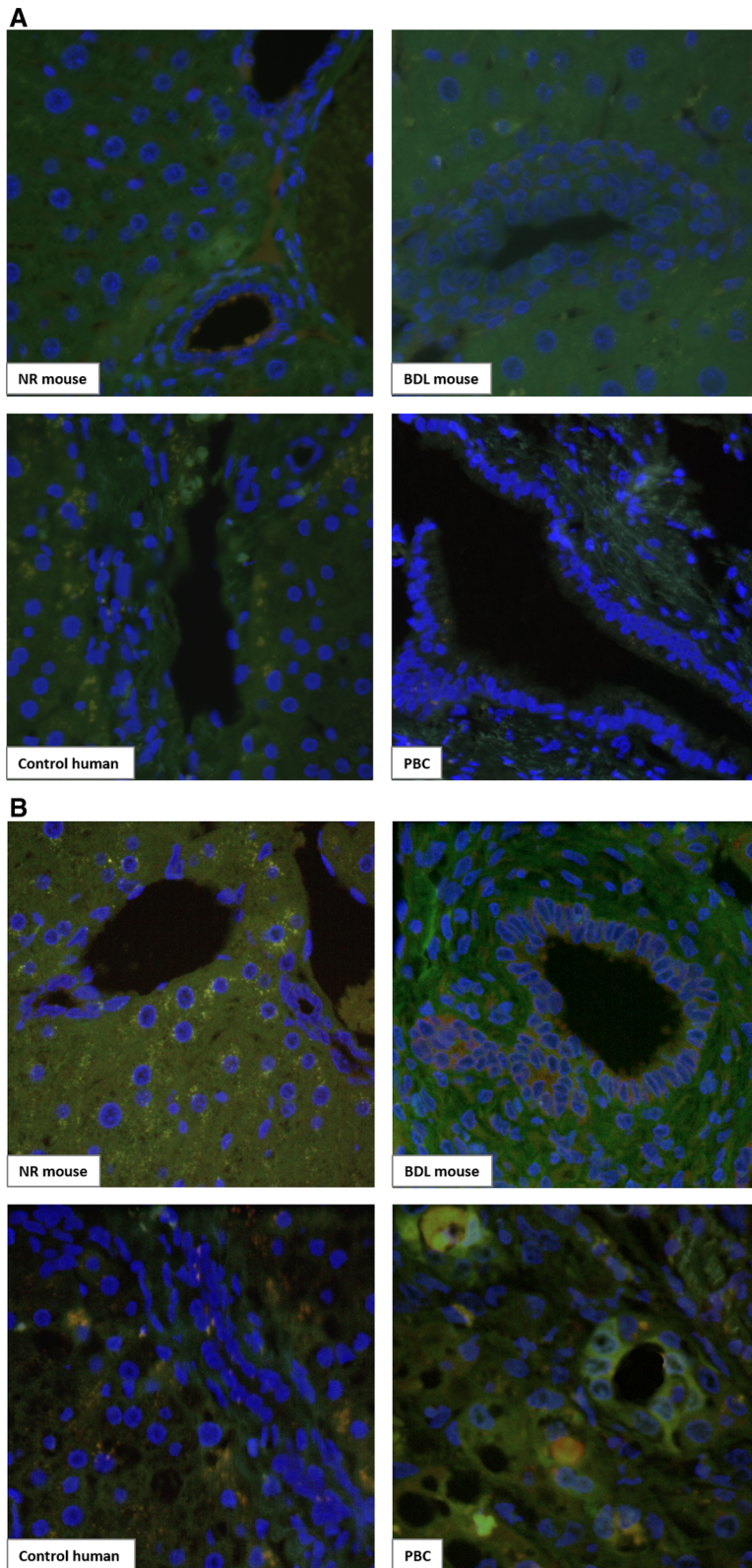
## Results

### Cholangiocytes express lactoferrin and its receptors

Immunohistochemistry in liver sections showed that bile ducts in normal and BDL mice are positive for Lf (Fig. 1a). The protein is also expressed in human specimens, both in normal subjects and in PBC patients. Its expression is increased in Stage IV PBC samples (Fig. 1a). Moreover, two receptors for Lf, LRP1 and ITLN1, at low and high specificity, respectively, have been detected. LRP1 presented a weak positivity in both normal and BDL mouse samples, whereas in human normal subjects it was negative. However, its expression is strongly enhanced in Stage IV PBC (Fig. 1b). ITLN1 was present both in normal and BDL mouse bile ducts. It was weakly expressed in bile ducts from human normal subjects and significantly increased in Stage IV PBC samples (Fig. 1c).

These results obtained with immunohistochemistry were confirmed by immunofluorescence in liver samples and in cells isolated from mice and in human biopsies. The co-localization of Lf and LRP1 in liver tissues has been evaluated. Lf (green) and LRP1 (Albar et al.) were not markedly co-localized in any sample (Fig. 2a). Lf (green) and ITLN1 (Albar et al.) were found to be co-expressed in bile ducts in BDL mouse and Stage IV PBC samples (Fig. 2b).

**Fig. 2** **a** Immunofluorescence to evaluate the co-localization of lactoferrin and LRP1 in murine and human livers. Specific immunoreactivity was shown in green for lactoferrin, in red for LRP1 and nuclei were stained with DAPI (blue). Lf and LRP1 were not significantly co-localized. **b** Immunofluorescence to evaluate the co-localization of lactoferrin and ITLN1. The co-localization was significant and markedly increased in bile ducts in BDL mouse and stage IV PBC samples. Specific immunoreactivity was shown in green for lactoferrin, in red for ITLN1 and nuclei were stained with DAPI (blue). Original magnification  $\times 40$ . (Color figure online)



In isolated cholangiocytes from mice and patients, we found co-localization of Lf and LRP1 in normal and BDL mouse cells. Furthermore, the co-localization is also present in cholangiocytes from Stage IV PBC samples, while being less present in the healthy cells (Fig. 3a). After LRP1, we studied the co-expression between Lf and the other receptor, ITLN1, in the same cells. Co-expression was increased both in BDL mouse and in PBC cholangiocytes (Fig. 3b).

The expression of Lf and ITLN1 by real-time PCR in total RNA from cholangiocytes of normal and BDL mice was also evaluated. The expression of Lf and ITLN1 was increased in BDL cholangiocytes compared to cells isolated from normal mice (Fig. 4).

#### Bovine lactoferrin increases cholangiocyte proliferation rate

In order to evaluate the effect of lactoferrin on cellular growth, cholangiocytes were treated with bovine Lf (bLf) (100 µg/ml) for 24 h. MTT proliferation assay showed a significant increase of the cellular proliferation rate when compared to control (Fig. 5). Cell growth was analyzed also by immunofluorescence for PCNA (Fig. 5). PCNA is a DNA clamp acting as a processivity factor for DNA polymerase in eukaryotic cells and is essential for replication. It is largely used as a marker of cellular growth. As shown in Fig. 5, bLf treatment enhances PCNA-positive cholangiocytes, supporting the results obtained by MTT assay.

## Discussion

The present study provides the following findings: (i) human and mouse cholangiocytes express Lf, which is present mostly in bile ducts of PBC patients; (ii) human and mouse cholangiocytes express Lf receptors: the low specificity receptor LRP1 and the high specificity receptor ITLN1. In particular, LRP1 results negative in normal human liver and over-expressed in human PBC samples, while it is weakly positive in normal and BDL mouse samples. ITLN1 is present in both normal and BDL mouse liver; in human liver, it is almost negative in bile ducts of normal subjects, while its expression is significantly increased in PBC patients. Moreover, bLf has a proliferative effect on cholangiocytes as shown by proliferation studies.

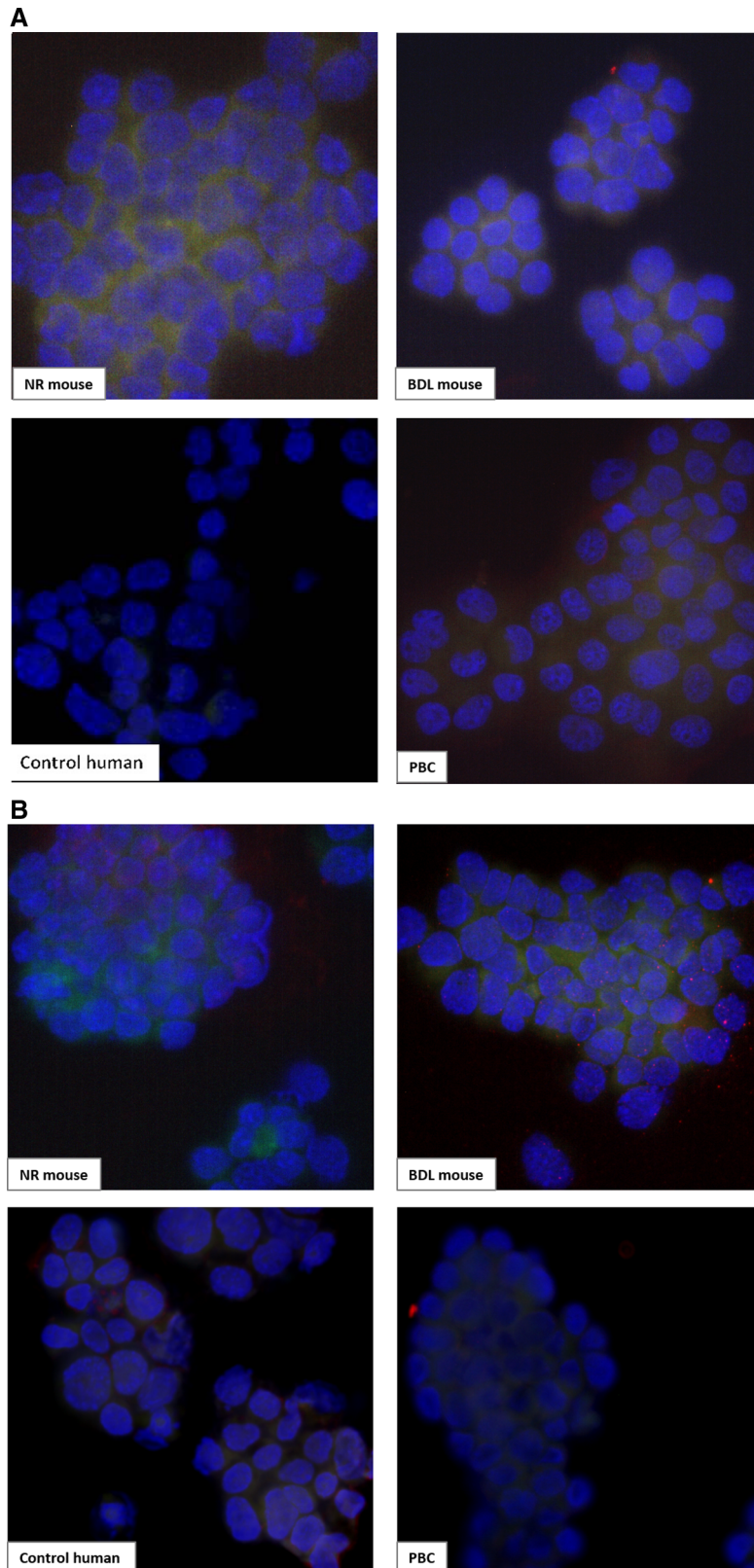
**Fig. 3** **a** Immunofluorescence in isolated cells to evaluate the co-localization of lactoferrin and LRP1. There was a low co-localization in all cell types. Specific immunoreactivity was shown in green for lactoferrin, in red for LRP1 and nuclei were stained with DAPI (blue). **b** Expression of lactoferrin and ITLN1 in isolated cholangiocytes. The co-localization was increased in BDL mouse and in PBC cholangiocytes compared to control cells. Specific immunoreactivity was shown in green for lactoferrin, in red for ITLN1 and nuclei were stained with DAPI (blue). Original magnification  $\times 40$ . (Color figure online)

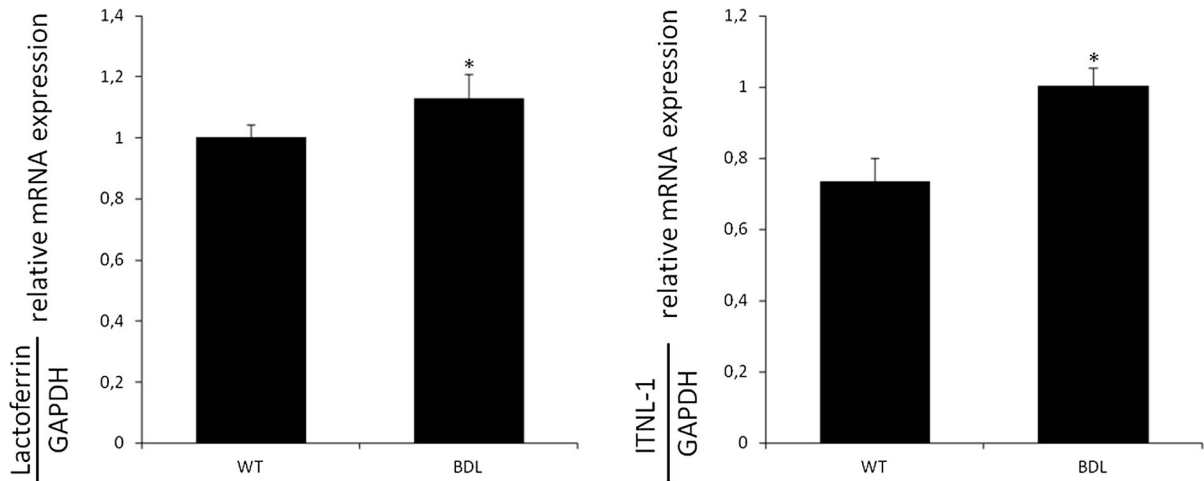
Up to now, the role of Lf in the liver has been studied in animal models in relation to injury and disease processes such as liver injury, obstructive jaundice and fibrosis (Taguchi et al. 2015). These studies have shown promising results to consider Lf as an hepatoprotective agent (Berlutti et al. 2011). To date, a role for Lf in cholangiocytes and pathologies involving the biliary epithelium has not been defined. The biliary epithelium is the target of the autoimmune response that characterizes PBC, a chronic autoimmune cholestatic disease. PBC samples show an intense inflammatory infiltrate which progresses from the portal space to involve the liver diffusely and culminates in fibrosis and cirrhosis in the advanced stages of the disease (Onori et al. 2007). Most studies demonstrated that apoptosis is a major mechanism of cholangiocyte death during PBC and the progression towards ductopenia is caused by a relative predominance of cholangiocyte apoptosis versus proliferation, that specifically occurs in the ductopenic PBC stage III and IV (Alvaro et al. 2004). Therefore, PBC is characterized by two important features: an intense inflammatory infiltrate and cholangiocyte death.

The results of this study, show that Lf is present in both human and murine cholangiocytes: in normal and BDL mice, human control and PBC samples. The receptors for Lf are present as well: in detail, the non-specific receptor LRP1 has been shown to be weakly present in normal and BDL mice, absent in normal human tissue, while it was significantly up-regulated in PBC samples; this seems to suggest an increased Lf uptake by diseased cholangiocytes. Similar results were observed for the Lf specific receptor ITLN1, whose expression in bile epithelium was also highly increased in PBC samples.

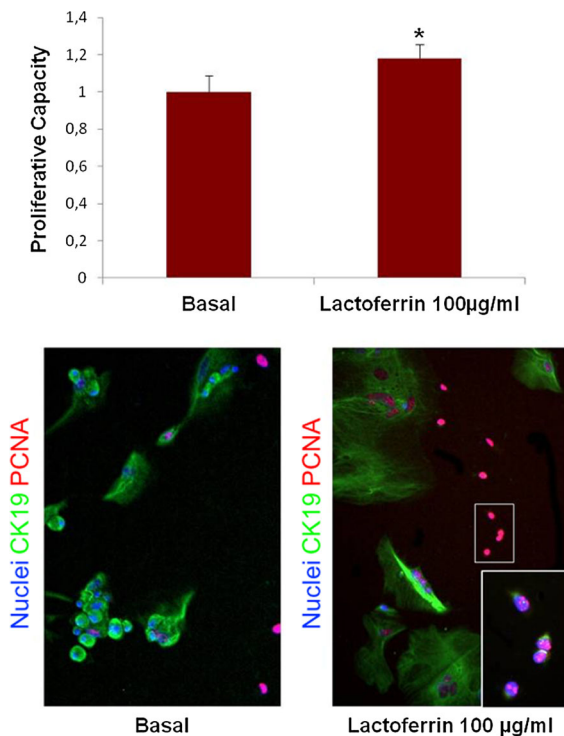
Once observed the presence of Lf and its receptors in the biliary epithelium, the potential effects of this protein in normal and pathological conditions have







**Fig. 4** By real-time PCR, the mRNA expression of lactoferrin and ITLN1 increased in BDL compared to normal cholangiocytes. Data are mean  $\pm$  SE of three evaluations. \* $P < 0.05$  versus the values of normal cholangiocytes



**Fig. 5** Effect of bovine lactoferrin on cholangiocyte proliferation (evaluated by MTT assay after 24 h of incubation). When non-malignant cholangiocytes were incubated with lactoferrin, there was a significant increase in proliferation. Data are mean  $\pm$  SE of 7 experiments. \* $P < 0.05$  versus its corresponding basal value. The bottom pictures show the co-expression of CK19 (green), a marker of biliary epithelial cell, and PCNA (Albar et al.), demonstrating that, after treatment with lactoferrin, there was an increase of PCNA-positive cells. Original magnification  $\times 40$

been evaluated. First, Lf showed a proliferative effect on biliary epithelium: data obtained through proliferation assays demonstrated that bLf treatment increased the proliferative capacity of cholangiocytes compared to the basal condition. Through immunofluorescence for PCNA, a factor essential for DNA replication, the co-localization between PCNA and CK7, a marker of cholangiocytes has been detected. The present data indicate that the biliary epithelium, by increasing the expression of Lf and its receptors display an attitude towards survival rather than apoptosis and that the up-regulation of Lf may prevent cholangiocyte apoptosis in the advanced stages of PBC.

In conclusion, Lf is emerging as an important bioactive molecule regulating cellular proliferation and differentiation, mediated by Lf receptors (Rosa et al. 2017). Therefore, bLf recognized by human Lf receptors may be considered as a promising nutraceutical and therapeutic agent in many fields, considering it is side effect-free, being a natural product. In our study, we have shown that bLf stimulates proliferation of biliary cells and this may represent an important factor for cholangiocyte survival in the terminal ductopenic stages of PBC and in other cholangiopathies. Moreover, it may be interesting to investigate the role of bLf in the inflammatory process, since the inflammatory cells present in the infiltrate express Lf, and that they supposedly worsen the general pathological condition.

## References

- Akiyama Y, Oshima K, Kuhara T, Shin K, Abe F, Iwatsuki K et al (2013) A lactoferrin-receptor, intelectin 1, affects uptake, sub-cellular localization and release of immunologically detectable lactoferrin by intestinal epithelial Caco-2 cells. *J Biochem* 154:437–448
- Albar AH, Almehdar HA, Uversky VN, Redwan EM (2014) Structural heterogeneity and multifunctionality of lactoferrin. *Curr Protein Pept Sci* 15:778–797
- Ali AH, Tabibian JH, Carey EJ, Lindor KD (2016) Emerging drugs for the treatment of primary biliary cholangitis. *Expert Opin Emerg Drugs* 21:39–56
- Alvaro D, Invernizzi P, Onori P, Franchitto A, De Santis A, Crosignani A et al (2004) Estrogen receptors in cholangiocytes and the progression of primary biliary cirrhosis. *J Hepatol* 41:905–912
- Berlutti F, Morea C, Battistoni A, Sarli S, Cipriani P, Superti F et al (2005) Iron availability influences aggregation, biofilm, adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. *Int J Immunopathol Pharmacol* 18:661–670
- Berlutti F, Superti F, Nicoletti M, Morea C, Frioni A, Ammendolia MG et al (2008) Bovine lactoferrin inhibits the efficiency of invasion of respiratory A549 cells of different iron-regulated morphological forms of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. *Int J Immunopathol Pharmacol* 21:51–59
- Berlutti F, Pantanella F, Natalizi T, Frioni A, Paesano R, Polimeni A et al (2011) Antiviral properties of lactoferrin—a natural immunity molecule. *Molecules* 16:6992–7018
- Carpino G, Cardinale V, Renzi A, Hov JR, Berloco PB, Rossi M et al (2015) Activation of biliary tree stem cells within peribiliary glands in primary sclerosing cholangitis. *J Hepatol* 63:1220–1228
- Carpino G, Renzi A, Franchitto A, Cardinale V, Onori P, Reid L et al (2016) Stem/progenitor cell niches involved in hepatic and biliary regeneration. *Stem Cells Int* 2016:3658013
- Cutone A, Frioni A, Berlutti F, Valenti P, Musci G, Bonaccorsi di Patti MC (2014) Lactoferrin prevents LPS-induced decrease of the iron exporter ferroportin in human monocytes/macrophages. *Biometals* 27:807–813
- Frioni A, Conte MP, Cutone A, Longhi C, Musci G, di Patti MC et al (2014) Lactoferrin differently modulates the inflammatory response in epithelial models mimicking human inflammatory and infectious diseases. *Biometals* 27:843–856
- Glaser SS, Gaudio E, Rao A, Pierce LM, Onori P, Franchitto A et al (2009) Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. *Lab Invest* 89:456–469
- Herz J, Strickland DK (2001) LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest* 108:779–784
- Ludwig J, Dickson ER, McDonald GSA (1978) Staging of chronic nonsuppurative destructive cholangitis (syndrome of primary biliary cirrhosis). *Virchows Arch A Path Anat Histo* 379(2):103–112
- Mancinelli R, Franchitto A, Glaser S, Meng F, Onori P, Demorrow S et al (2013) GABA induces the differentiation of small into large cholangiocytes by activation of Ca(2+)/CaMK I-dependent adenylyl cyclase 8. *Hepatology* 58:251–263
- Onori P, Alvaro D, Floreani AR, Mancino MG, Franchitto A, Guido M et al (2007) Activation of the IGF1 system characterizes cholangiocyte survival during progression of primary biliary cirrhosis. *J Histochem Cytochem* 55:327–334
- Rosa L, Cutone A, Lepanto MS, Paesano R, Valenti P (2017) Lactoferrin: a natural glycoprotein involved in iron and inflammatory homeostasis. *Int J Mol Sci* 18:1985
- Sessa R, Di Pietro M, Filardo S, Bressan A, Rosa L, Cutone A et al (2017) Effect of bovine lactoferrin on *Chlamydia trachomatis* infection and inflammation. *Biochem Cell Biol* 95:34–40
- Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL (2009) Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. *Sci Signal* 2:ra18
- Suzuki YA, Lopez V, Lonnerdal B (2005) Mammalian lactoferrin receptors: structure and function. *Cell Mol Life Sci* 62:2560–2575
- Suzuki YA, Wong H, Ashida KY, Schryvers AB, Lonnerdal B (2008) The N1 domain of human lactoferrin is required for internalization by caco-2 cells and targeting to the nucleus. *Biochemistry* 47:10915–10920
- Taguchi K, Yamasaki K, Seo H, Otagiri M (2015) Potential use of biological proteins for liver failure therapy. *Pharmaceutics* 7:255–274
- Ward PP, Paz E, Conneely OM (2005) Multifunctional roles of lactoferrin: a critical overview. *Cell Mol Life Sci* 62:2540–2548
- Wei M, Guo X, Tu L, Zou Q, Li Q, Tang C et al (2015) Lactoferrin-modified PEGylated liposomes loaded with doxorubicin for targeting delivery to hepatocellular carcinoma. *Int J Nanomed* 10:5123–5137
- Wrackmeyer U, Hansen GH, Seya T, Danielsen EM (2006) Intelectin: a novel lipid raft-associated protein in the enterocyte brush border. *Biochemistry* 45:9188–9197
- Zhang Y, Lima CF, Rodrigues LR (2014) Anticancer effects of lactoferrin: underlying mechanisms and future trends in cancer therapy. *Nutr Rev* 72:763–773