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Tesi di Laurea: "Role of lactoferrin and its receptors on biliary epithelium and activation of hepatic progenitor cells" (Sintesi)

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Background

Lactoferrin (Lf) is a multifunctional iron binding glycoprotein of 692 amino acids, produced by exocrine glands and neutrophils (Berlutti et al. 2005). This glycoprotein belongs to the transferrin family and has a highly conserved structure among human, bovine, mouse, and other species (Berlutti et al. 2011). Many studies have unraveled the numerous functions of Lf: host defense, antimicrobial and anti-inflammatory activities, iron homeostasis, cellular growth and differentiation, and anti-cancer activity (Frioni et al. 2014, Sessa et al. 2017, Zhang et al. 2014). Other than by chelating iron, Lf 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 Lf uptake from the digestive tract; 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 Lf, possibly via activation of cell signaling pathways. Another LfR is the LDL receptor related protein 1 (LRP1), which is a low specificity receptor for Lf, 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 Lf, 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). The regenerative capability of the liver is well known since ancient times. We now know stem or progenitor liver cells are present in niches in the liver, along the biliary tree and in the liver acini (Carpino et al. 2016). In particular, stem cells known as hepatic progenitor cells (HPCs), which are precursors only to hepatocytes or cholangiocytes are present in the Canals of Hering. They participate in the small intrahepatic bile duct renewal (Carpino et al. 2016). Other than these cells, niches also contain precursors to other cells and a connective tissue matrix rich in hyaluronans, proteoglycans, fetal collagens and laminin. HPCs can differentiate into hepatocytes or cholangiocytes both in human and rodents. HPCs are positive for markers such as SOX9, SOX17, CK7, CK19, EpCAM, NCAM. These cells can self-renew or differentiate. The niche contributes to liver cell turnover and it is also activated in liver disease (Carpino et al. 2016). In this circumstance, when proliferative capabilities of mature liver parenchymal cells become exhausted by chronic damage and prolonged cell death, HPCs proliferate, giving rise to a ‘ductular reaction’ (DR), so called because it consists of ‘reactive ductules’ that are cell strings with irregular lumina. The majority of chronic liver disease (such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis, PBC) and also acute conditions, such as liver failure, show evidence of DR. The DR can have a different phenotype based on the etiology of the underlying disease. For example, in PBC, it is mostly composed of cells expressing biliary (cytokeratin 7 and 19), neuroendocrine (NCAM, Chromogranin A), and stem cell markers (SOX9 and CD133). Instead, in liver diseases of non-biliary origin, they express hepatocyte-related characteristics (Carpino et al. 2016).

Primary biliary cholangitis (PBC) is an autoimmune 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 peri-portal 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:

1) to evaluate the expression of Lf and its receptors on cholangiocytes in vivo and in vitro both in human samples (normal subjects and PBC patients) and in mouse (normal and BDL)
2) to evaluate its role in the process of activation of the hepatic progenitor cells (HPCs)

Main materials and methods

Reagents were purchased from DBA ITALIA srl (Milan, Italy), unless otherwise indicated. Highly purified bovine lactoferrin (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). Animal model used were male C57/BI6 N mice (20–25 g) 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 animals were used for the harvest of tissues and purification of liver cells. Human samples in the study were from liver biopsies of 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.

Pure cholangiocytes 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. For the *in vitro* experiments, we used a line of human cholangiocyte stem cells obtained from CellProgen (M36755–11S), maintained in its specific medium as model of HPCs. All these cells have been used for the immunofluorescence, inflammation evaluation, and MTT cell proliferation assay.

Immunohistochemistry (IHC) and immunofluorescence (IF)

Specimens were fixed in 10% buffered formalin, embedded in paraffin and cut in sections of 3 μm. For IHC, endogenous peroxidase activity was blocked by incubation in hydrogen peroxide. Sections were then incubated over-night at 4°C with the specific primary antibodies. Samples were rinsed twice with PBS, incubated at room temperature with secondary biotinylated antibody and then with Streptavidin-HRP. Diaminobenzidine 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 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 and counterstained with 4, 6-diamidino-2- phenylindole (DAPI) for visualization of cell nuclei. Smears/Cultures were 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^2 Real-Time assay from SABiosciences (Frederick, MD). A DDC_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 ± SE of Lactoferrin and Intelectin-1 to GAPDH ratio.

Proliferation evaluation

After trypsinization, HPCs were seeded into 96-well plates (10,000 per well) in a final volume of 200 µl of growth medium and allowed to adhere to the plate overnight. Cells were stimulated with bovine lactoferrin (100 µg/ml) at 37°C for 24 hours before evaluation of cell growth through the Cell Proliferation Assay kit, used for fast and sensitive quantification of cell proliferation and viability. 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.

Inflammation evaluation

As described in the introduction section, lactoferrin has been studied as an important immunomodulating agent with anti-inflammatory properties. For this reason, we evaluated the levels of Interleukin 6 (IL-6) in the supernatants of the HPCs with or without treatment with lactoferrin. The amount of IL-6 secreted by cholangiocytes was evaluated through an ELISA kit. Briefly, HPCs were seeded into 6-well plate (500,000 per well) in a final volume of 2 ml of growth medium and allowed to adhere to the plate overnight. Cells were stimulated with or without bovine lactoferrin (100 µg/ml) for 48 hours and incubated at 37°C. The day after, the supernatants were collected and transferred to a tube and stored at −70°C before analysis for IL-6 levels by ELISA using commercially available kits

(AVIVA system biology). The IL-6 ELISA Kit is based on standard sandwich enzyme-linked immune-sorbent assay technology. The density of yellow coloration read by absorbance at 450 nm and is quantitatively proportional to the amount of sample human IL-6 captured in the well.

Statistical analysis

Data are presented as arithmetic mean ± 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.

Results

Cholangiocytes express lactoferrin and its receptors

Immunohistochemistry in liver sections showed that normal and BDL mice bile ducts are positive for lactoferrin. The protein is also present in human tissue, both in control and PBC patients. Its expression is increased in stage IV PBC samples. We considered two receptors for lactoferrin: LRP1, a low specificity receptor, and ITLN1, a high specificity receptor. The former presented a weak positivity in mouse samples, whereas in human controls it was negative. However, its expression is strongly enhanced in stage IV PBC. ITLN1 was present both in normal and BDL mouse bile ducts. It was weakly expressed in bile ducts from human control samples and significantly increased in stage IV PBC samples. The results obtained with immunohistochemistry were confirmed by immunofluorescence in liver tissue and in cells isolated from mice and patient biopsies (images can be found on the full version of this thesis).

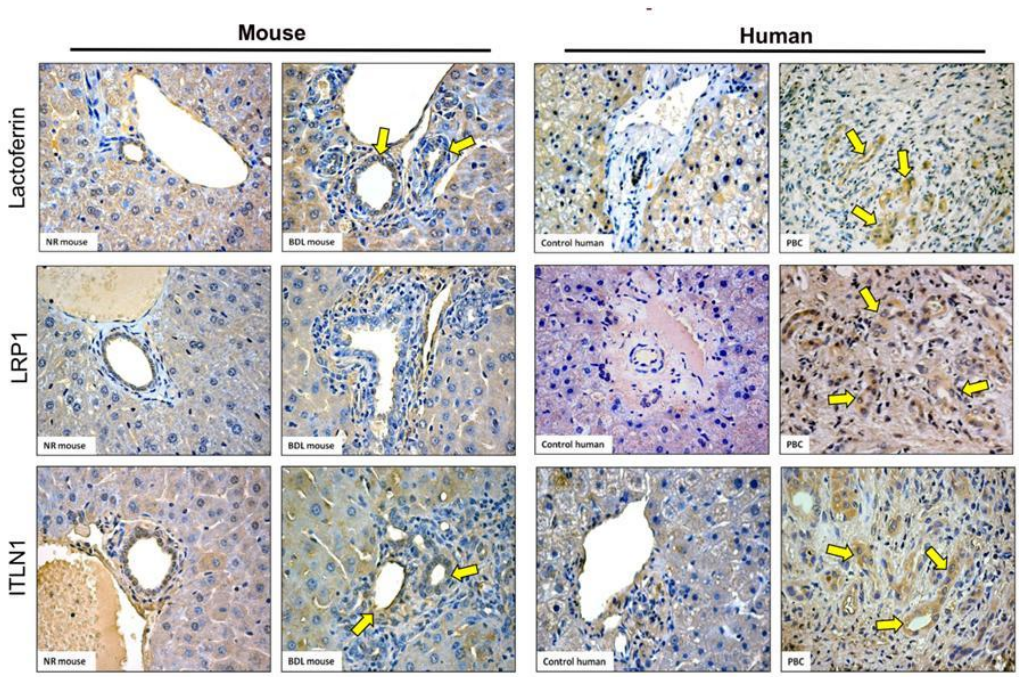

Figure 1. Immunohistochemistry in liver sections. Antigens looked for are shown on the side, species of the tissue is shown on top. Arrows indicate increased expression. Original magnification x40.

The expression of lactoferrin and ITLN1 was also evaluated by real-time PCR in total RNA from cholangiocytes of normal and BDL mice. The expression of lactoferrin and ITLN1 was increased in BDL cholangiocytes compared with cells from normal animals (Fig.2).

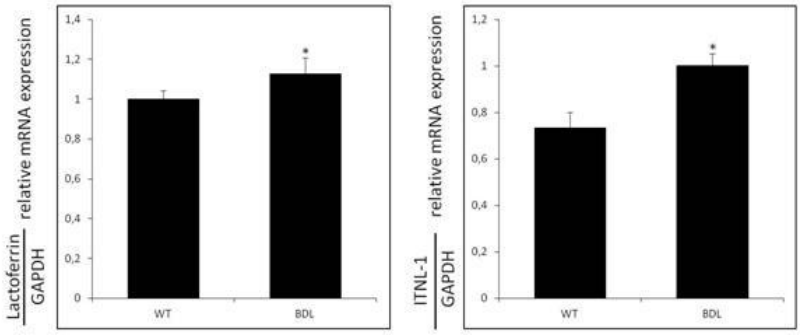

Figure 2. By real-time PCR, the mRNA expression of lactoferrin and ITLN1 increased in BDL compared to normal cholangiocytes. Data is reported as a mean ± SE of 3 evaluations. *P <0.05 vs. the values of normal cholangiocytes.

Evaluation of proliferation

Treatment with lactoferrin (100 µg/ml) significantly increased the growth of HPCs after 24 hours of incubation compared to controls, as shown by MTT cell proliferation assay and PCNA expression (Fig.3). PCNA, proliferating cell nuclear antigen, 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. PCNA is marked in red, and cytokeratin 19, a marker of biliary epithelial cell, in green: there is an increase of PCNA-positive cholangiocytes after treatment with lactoferrin, supporting the results obtained from the MTT assay (Fig.3).

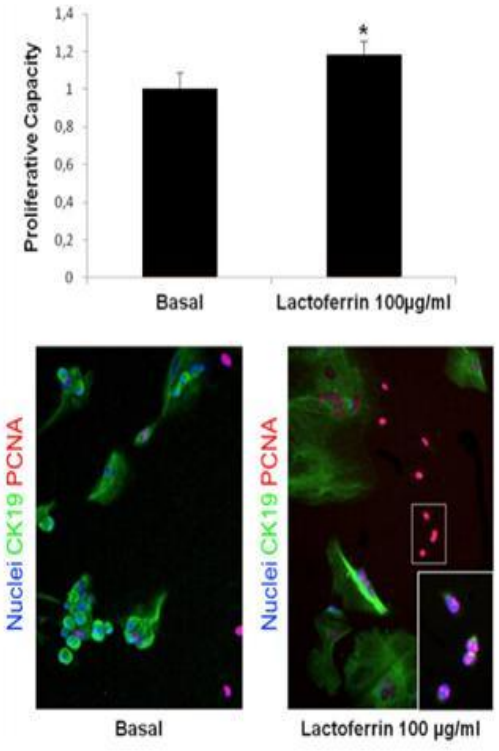

Figure 3. Effect of lactoferrin on HPCs proliferation (evaluated by MTT assay after 24 h of incubation). When cholangiocytes were incubated with lactoferrin, there was a significant increase in proliferation. Data is reported as a mean ± SE of 7 experiments. *P 0.05 vs. its corresponding basal value. The bottom pictures show the co-expression of

CK19 and PCNA, demonstrating that, after treatment with lactoferrin, there was an increase of PCNA-positive cells. Original magnification x40.

Evaluation of inflammation

We demonstrated that, after long-term cultures (24 and 48 hours), HPCs secrete IL-6 after administration of LPS (especially after 48 hours), a pro-inflammatory cytokine. We found a significant decrease in IL-6 secretion in HPCs if, after stimulation with LPS for 3 hours, the cells were treated with bovine lactoferrin for 48 hours, compared to treatment with LPS only (Fig.4). The data supports the role of lactoferrin in modulating inflammation by an autocrine mechanism.

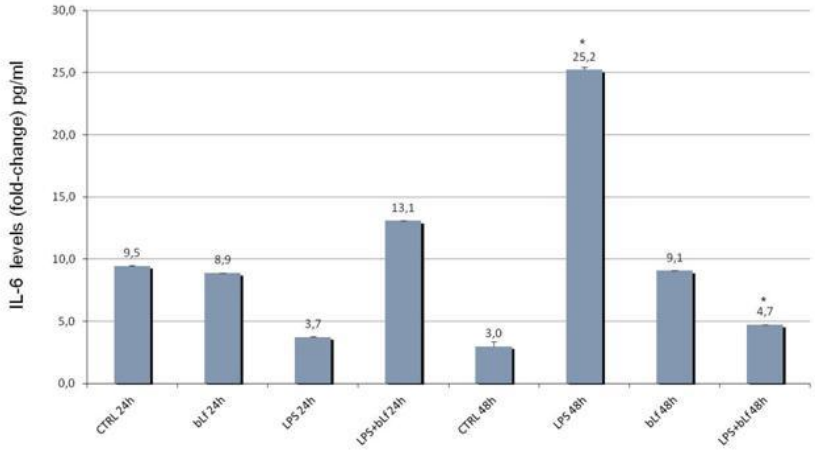

Figure 4. Evaluation of IL-6 levels in the supernatant of HPC cultures (24 hours and 48 hours). The administration of LPS for 48 hours induces inflammation in epithelial cells and secretion of IL-6. The addition of bovine lactoferrin (bLF) decreased the levels of IL-6 compared to LPS only. Data is reported as a mean ± SE of 7 evaluations. **P* 0.05 vs. control levels of human cholangiocytes.

Evaluation of hepatic progenitor cells activation and of lactoferrin expression in HPCs

Immunohistochemistry for CK7, a cytokeratin expressed by cholangiocytes and HPCs, showed that in control human liver sections, CK7+ cells line the bile ducts (Fig.5A). CK7 expression is strongly enhanced showing an intense DR in PBC samples (Fig.5B).

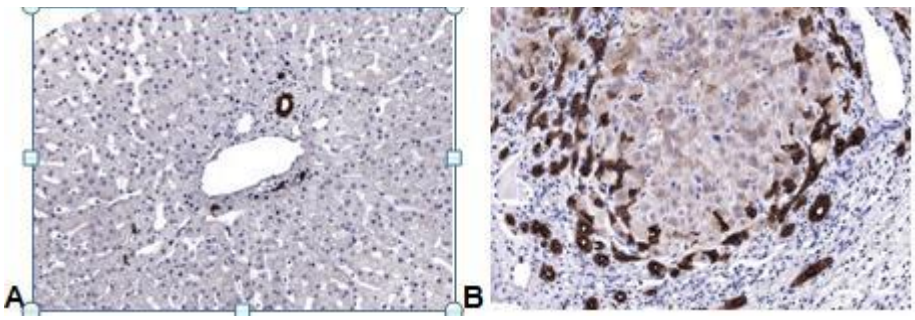

Figure 5. Immunohistochemistry for cytokeratin 7 (CK7) in a control sample **(A)** compared to tissue from a liver biopsy of a stage IV PBC patient **(B)**. Original magnification x20.

Immunofluorescence for CK7 and SOX9, a specific marker of HPCs, showed co-expression in the bile ducts and an intense ductular reaction in PBC samples.

After investigating HPC activation, we assessed the presence of lactoferrin in the reactive ductules of PBC, evaluating its co-expression with a cocktail of cytokeratins (pan-CK) expressed by cholangiocytes and, subsequently, its co-expression with SOX9, a marker of HPCs. IF showed co-expression in both cases.

After investigating the co-expression of Lf with markers of HPCs, we also evaluated the co-expression of LRP1 and CK7 in normal and PBC bile ducts, and the co-localization of LRP1 with SOX9. Except for the LRP1/SOX9 co-expression in normal tissue, in all other samples we found a stronger co-expression, particularly in stage IV PBC samples. We performed a similar study to evaluate the co-expression of ITLN1, a highly-specific lactoferrin receptor, in normal and PBC liver samples, with CK7 and EpCAM, another marker for HPCs. ITLN1 is intensely expressed in stage IV PBC samples and it is co-expressed both with CK7 and, even more, with EpCAM, confirming a possible role for Lf in the activation of HPCs.

In the last part of the study, we assessed the presence of inflammatory cells in stage IV PBC samples, and the co-expression with Lf. We used CD68 and S100A9 as markers of macrophages and Ly6G as a marker of neutrophils. In PBC samples, macrophages and neutrophils were present, although mainly macrophage markers showed co-expression with Lf.

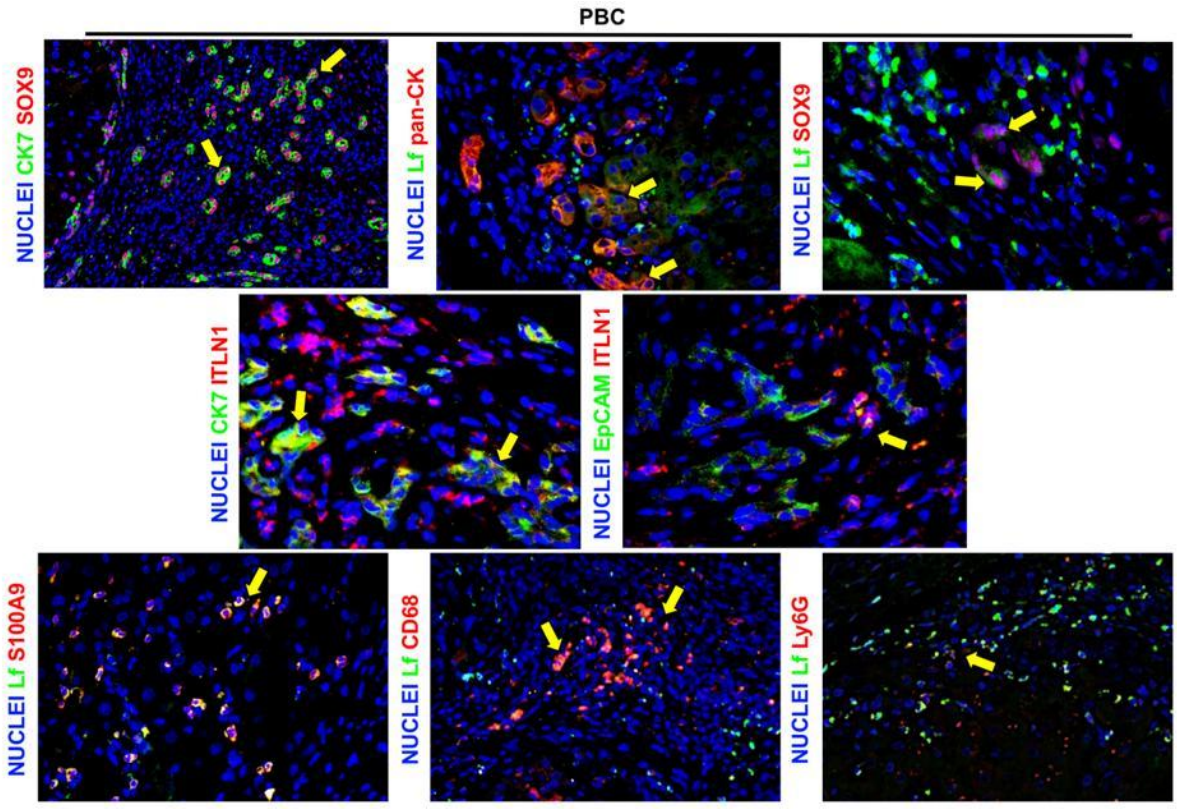

Figure 6. Immunofluorescence in liver sections of PBC patients to study activation of HPCs and expression of Lf and its receptors within them and the DR. Arrows indicate increased expression. Original magnification x40.

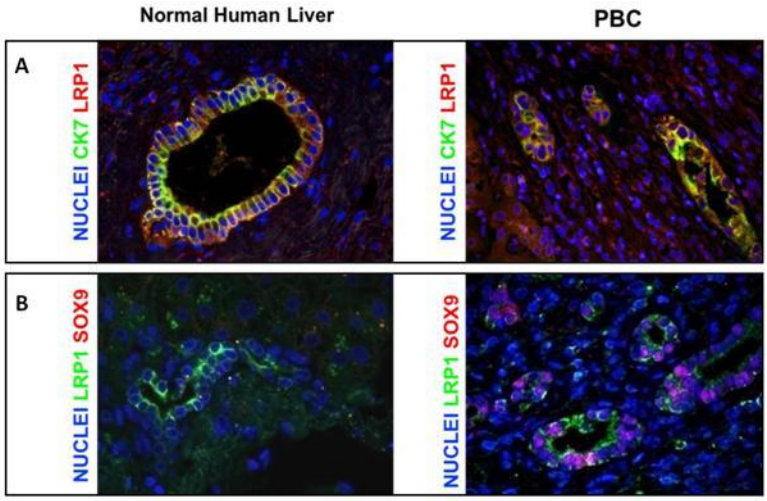

Figure 7. Immunofluorescence to evaluate co-expression of CK7 and LRP1, the low specificity receptor for lactoferrin (A - upper panel), and co-expression of SOX9 and LRP1 (B - lower panel) in normal and stage IV PBC liver samples. Original magnification x40.

Discussion

The present study provides the following findings:

1) Human and mouse cholangiocytes express lactoferrin, which is present mostly in cholangiocytes in liver sections from PBC patients
2) Human and mouse cholangiocytes express lactoferrin receptors: both LRP1 and ITLN1 expression is significantly increased in PBC cholangiocytes
3) Lactoferrin has proliferative and anti-inflammatory effects
4) The HPC compartment is activated in PBC in comparison with normal human liver tissue. Lf expression is increased in pan-CK+ DR in PBC compared to bile ductules in normal liver samples and it is co-expressed with SOX9 in cells within DR, supporting the hypothesis that lactoferrin is involved in the activation of HPCs
5) In PBC, both LRP1 and ITLN1 are co-expressed within the DR with HPC markers
6) The number of CD68+/Lf+, S100A9+/Lf+ and Ly6G+/Lf+ cells is increased in PBC compared to normal liver samples, indicating that the inflammatory cells present in the PBC infiltrate secrete Lf, that may be involved in the modulation and control of the inflammatory process

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 ITNL1, 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 been evaluated. First, Lf showed a proliferative effect on HPCs: 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.
We also investigated the anti-inflammatory effect of lactoferrin, known to have immunomodulatory properties: our study confirmed this by showing lactoferrin to have an anti-inflammatory action in HPCs treated with LPS to induce inflammation, which was quantified by expression of IL-6, a pro-inflammatory cytokine. The cells to which lactoferrin was subsequently added showed a significant reduction in IL-6 at 48 hours.
We also confirmed that the HPC compartment is activated in this disease process and we showed that the DR characteristic of HPC activation is positive for stem cell markers and that lactoferrin expression is increased in pan-CK+ DR in PBC patients compared to normal livers. Lactoferrin was also shown to be co-expressed in cells positive for stem cell markers. Furthermore, lactoferrin seems to be present abundantly in the inflammatory cells making up the infiltrate characteristic of PBC – as shown by IF studies, probably acting not only in an autocrine but also in a paracrine pathway to enhance its effects on cholangiocytes, the target cells of the disease process in 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 has an anti-inflammatory effect on them; 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.

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Sintesi della Tesi di Laurea discussa il 10 luglio 2017

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