Лактоферрин та захворювання порожнини рота: сучасний стан та перспективи при пародонтиті

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Загальна інформація

Лактоферрин (Lf) - глікопротеїн, що зв'язує залізо, здатний утворювати два іони заліза на молекулу, є компонентом секрецій людини, що синтезуються екзокринними залозами та нейтрофілами в місцях інфікування/запалення. Лак-. тоферин у слині є важливим фактором захисту від бактерій включаючи ті, що відносяться до Streptococcus mutans та бактерії парадонтопатії із його здатністю зменшувати ріст бактерій, розвиток біоплівки, перенасичення залізом, реакційне утворення кисню та запальні процеси.

Відповідне регулювання синтезу прозапальних цитокінів може бути корисним для зменшення деструкції тканини пародонту. Так, з'явився мультифункціональний IL-6, як важливий чинник, здатний модулювати кістку, залози та запальний гомеостаз.

Наразі ми повідомляємо про огляд функцій Lf, а також про протизапальну здатність Lf проти пародонтиту у моделі in vitro та клінічне спостереження.

У моделі in vitro, представленої дерматологічними фібробластами, інфікованими Prevotella intermedia, Lf показала сильну протизапальну активність. У спостережувальному клінічному дослідженні, проведеному за допомогою губного Lf (bLf), що вводять місцево добровольцям, що страждають періодонтитом, bLf знижують цитокіни, в тому числі IL-6 в кишковій рідині, набряках, кровотечі, глибинних кишенях, гінгівальних індексах та бляшках, тим самим поліпшуючи клінічні рівні. Навіть якщо потрібні інші клінічні випробування, ці результати дають суттєві докази на користь терапевтичного потенціалу цього багатофункціонального природнього

Ключові слова: лактоферин, порожнина рота, періодонтит, зараження.

Введення

Лактоферин (Lf), 80-kDa залізо-зв'язуючий глікопротеїн, що належить до сімейства трансферину, є компонентом секреції людини, включаючи слину, і синтезується екзокринними залозами та нейтрофілами в місцях інфікування та запалення (1). Lf, що містить 691 амінокислотний залишок, складається у дві гомологічні частки (залишки N-частки1-333 та залишки С-частки 345-691), з'єднані пептидом (залишок 334-344), який утворює 3-спіраль-петлю (рис.1). Цей глікопротеїн є високо консервативним серед людей, а також коров'ячих, миш'ячих та свинячих видів, здатний оборотним чином хелатувати два іони заліза для однієї молекули з високою спорідненістю (Kd ~ 10-20 M), що утримує вміст залози із значенням рН до 3,0, в той час як трансферрин зберігає залізо до приблизно рН 5,5 (1, 2, 3). Залізозв'язуючість досить висока, і за наявності Lf або трансферина концентрація вільного заліза в органічних рідинах не може перевищувати 10 ~ 18 M. Таким чином, запобігаючи преципітації цього металу як нерозчинних гідроксидів, інгібуючи ріст мікробів та перешкоджаючи утворенню видів реактивних оксигенів та пов'язаних з ними запальних процесів (1). Концентрація Lf у екзокринних секреціях людини описана в Таблиці 1. Заявлені концентрації збільшуються в місцях інфікування та/або запалених ділянках згідно розкриття нейтрофілів: 106 нейтрофілів виділяють 15 pg Lf. Антибактеріальна активність лактоферрина пов'язана і не пов'язана із здатністю утримання заліза. Перша функція, що приписується Lf, була антибактеріальною активністю в залежності від його здатності приєднувати залізо, що необхідно для росту і виживання бактерій (5). Ця дія Lf розглядалася як бактеріостатична і оборотна шляхом додавання гемосидерину (6).
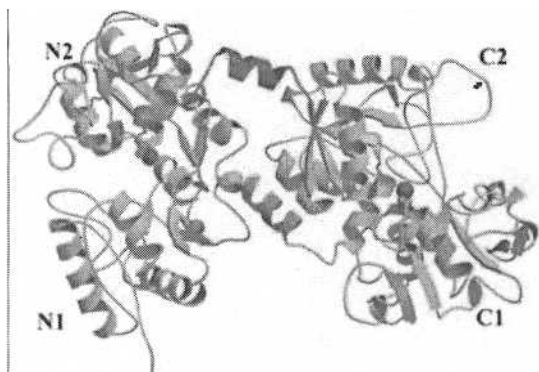
Рис 1 – Структура лактоферрину. Взято з: Baker & Baker, 2004 (4).

Таблиця 1 – лактоферрину концентрація у секреціях людини

Біологічні рідини	Концентрація (мг/мл)
Колострум	8
Молоко	1.5-4
Сльози	2
Слина	0.008
Синовія	0.001
Вагінальний секрет	0,008
Сперма	0.112
ЦСР	Не визнач.
Плазма	0.0004

Проте, бактеріальні збудники здатні подолати брак заліза за допомогою двох основних систем. Перший представлений синтезом малих хелаторів, сидерофорами, які зв'язують іони заліза з високою спорідненістю і переносять в бактерії через специфічний рецептор (7, 8). Окрім синтезу сидерофорів, деякі з бактеріальних видів, адаптованих до хазяїна, таких як Porphyromonas gingivalis, безпосередньо отримують через поверхневі рецептори, і здатні специфічно зв'язувати Lf або інші молекули, що зв'язують залізо, як гемін і гем (9,10,11), і транспортувати його через зовнішню мембрану. Потім залізо зв'язується периплазматичним залізо-зв'язуючим білком FbpA і транспортується в бактерії через внутрішній мембранний комплекс, що складається з FbpB та FbpC (12).

Залізо-незалежний бактерицидний ефект Lf також був описаний (13). Для безпосереднього впливу потрібна пряма взаємодія між Lf та ліпополісахаридом (LPS) грамнегативних або ліпотехіоевої кислоти грампозитивних бактерій | 14, 15, 16). Furthermore, it has been demonstrated that the bin¬ding of Lf to the lipid A of LPS (17, 18), induces, a release of LPS. This bactericidal activity of Lf appears to be loca¬ted in the N-terminal region as its derivative cationic pep¬tide (residues 17-41), called lactoferricin (Lfcin), is several fold more active than the intact protein (19, 20, 21). More recently, the 1-11 residues in N-terminal region of Lf has shown to possess an anf|bacl6isractivity against Strep-tococcus mutans through its binding with bacterial DNA (22). Moreover, the Lf antibacterial activity related to its capa-bility to release LPS can be annulled by high calcium con-centration in the culture media (23). As Lf is also able to bind Cafll) through the carboxylate groups of the sialic acid residues, present on two glycan chains, it cannot be ruled out that the release of LPS from Gram-negative bacteria can be also due to this additional binding property of Lf (23).

Lactoferrin in saliva
Saliva is composed of 98% water and its pH is around 6.64 (24, 25). Saliva composition consists of hormones, peptides, electrolytes, mucus, antibacterial compounds and various enzymes (26). Steroid hormones detectable in saliva comprise cortisol, androgens including testosterone and dehydroepiandrosterone, oestrogens and progesterone as well as aldosterone. Furthermore, saliva is rich in organic constituents as proteins, albumin, urea, uric acid, lactate, and creatinine (26). Inorganic compounds are also present in saliva; Na^+, K^+ and Ca^{2+} are the main cations and CL and HCO_3^-

kers of mucosal immunity as immunoglobulins, including IgA, igM and IgG, cx-amylase, lysozyme and Lf are detectable in saliva (27). Evidence is constantly increasing to support the use of saliva as a non-Invasive tool for monitoring biomarkers in health and pathological human status (28).
However, it is important to follow some guidelines prior to saliva collection in order to minimize error variance and chances of methodological errors (29, 30). Food or drink intake must be avdideci at least 2 h prior to sampling, due to variatidiis in Saliva secretion which in turn will negatively affect the results. Food or drink high in sugar content, caffeine or acidity can stimulate saliva flow, and acidity will lower mouth pH levels, both compromising antibody-antigen binding and enzyme activity thus leading to invalid immunoassay results. In addition, alcohol consumption 24 h prior to sampling should be avoided as it may cause increased saliva secretion (29, 30, 31).
Among salivary proteins, Lf is the most important factor of natural immunity. Its concentration corresponds to 1.23 mg/l in gingival crevicular fluids (GCF) and to 8.96 and 7.11 mg/l in unstimulated and stimulated saliva, respectively. The Lf concentration in oral cavity is related to different fluid samples to be assayed. In fact, unstimulated saliva mainly derives from *70%* submandibular, 20% parotid, and 2% sublingual glands, while stimulated saliva mainly derives from 30% submandibular and 60% parotid glands (28). Moreover, the concentration of this glycoprotein in GCF is influenced by the amount of the excreted fluid which, in turn, depends from physiological or pathological status of the subject. The Lf concentration, measured /site, clearly shows that its levels increase from 36 ng/site in healthy humans to 63 and 90 ng/site in gingival and periodontal diseases, while its concentration calculated /ml is similar in health and pathological conditions (32).
Even if the detection of Lf as well as of other proteins in saliva, easily accessible source of potential local and systemic biomarkers of health and pathological status, the different amount of salivary flux in calculating the real concentration of the proteins must be considered.
This is a very critical point which can explain the conflicting data reported by different Authors on Lf oral concentration. Of note, several defence proteins (e.g. lysozyme, Lf and histatin-1) were found significantly more abundant in old (55-65 years old) than in young female (20-30 years old) subjects (33). These results, demonstrating that Lf synthesis in the saliva of females is age dependent, are in sharp contrast with the well ascertained data showing

that Lf synthesis is under the control of steroid hormones (34) and consequently, it decreases in menopausal and postmenopausal periods.

Summarizing, the changes of the Lf concentration depend not only from significant changes of saliva samples (unstimulated, stimulated) but they are also age-dependent. It was reported that Lf levels in GCF of patients with gingivitis, adult periodontitis (chronic periodontitis), and localized juvenile periodontitis (aggressive periodontitis) are similar, but higher than in normal subjects (32,35,36). Another study indicates that Lf level in GCF correlates with the clinical severity of periodontal diseases and the number of polymorphonuclear leucocytes (37). A decrease in Lf levels was observed in GCF and saliva after surgical periodontal treatment In chronic periodontitis (38). Similar Lf decrease in GCF and peripheral blood after oral hygiene procedures in experimental gingivitis using healthy volunteers was also detected (39). Lf is not synthesized in the healthy gingival tissues and elevated Lf levels in the GCF of inflamed tissues originate from invading inflammatory cells (40). Thus, Lf is released from neutrophils in GCF in response to the inflammatory condition of periodontitis as a potential host defence factor against perio- dontopathic bacteria and may be a good marker of periodontal diseases.

Influence of iron and lactoferrin on the lifestyle of oral microbiota

In human saliva, the iron content ranges from 0.1 to 1.0 pM depending on meals but it can increase for gingiva! bleeding due to infection and inflammatory processes. During the infection and inflammatory processes, the recruitment of neutrophils increases saliva Lf concentration from 20 to 60 pg/ml (41).

Therefore, saliva represents an interesting mode! to investigate the influence of iron and Lf concentrations on bacterial infections. As matter of fact, the different ratio between iron and Lf plays an important role in the lifestyle of several bacteria (42, 43) by inducing aggregation and biofilm development (41). in particular, in a saliva pool well defined for iron and Lf content, apo-Lf (iron unsaturated form) was found to enhance *S. mutans* aggregates and biofilm formation, whereas iron-saturated Lf decreased aggregation and biofilm developmenl). Similar behaviour has been recently described in the periodontopathogen *Ag- gregatibacter (Actinobacillus) actinomycetemcomitans:* iron limitation up-regulates its biofilm genes contributing to biofilm formation (44).

The reported data suggest that to assess the effect of Lf in oral cavity it is necessary to evaluate preliminarily the iron content of saliva. In fact, in periodontitis patients, the high iron concentration and the presence of hemin, which can form complexes with Lf, together with Lf degradation by bacterial and human enzymes (45, 46), could be responsible, in vivo, for the lack or reduced activity of Lf even if its concentration is increased following infection and inflammation.

Inhibition of bacterial adhesion on abiotic and cell surfaces by lactoferrin

Abiotic surfaces

Microbial adhesion and subsequent colonization, resulting in biofilm formation on abiotic surfaces such as dental surfaces and medical devices as dental prosthesis, represents

both a physiological process and a serious problem that can lead to oral illness. Efforts to control microbial adhesion by anti-adhesive new materials or compounds have had modest success once applied to the patient. Consequently, it could be very helpful to discover other compounds able to hinder microbial adhesion. The ability of Lf, in both apo- and iron-saturated form, to inhibit the adhesion of *S. mutans* to hydroxyapatite (HA), mimicking tooth surface (47), may represent an interesting function. The demonstration that Lf inhibits the adhesion of *S. muions* to a salivary film and HA through residues 473-538 of its C-lobe (48), further helped to understand this activity, which is unrelated to its iron binding properties. Both apo- and iron-saturated bLf also inhibit adhesion of free and aggregated *S. mutans* cells to a dental polymer when Lfs were pre-coated to dental polymer or bound to both dental polymer and bacterial cells (41). Apo-Lf but not iron-saturated Lf also inhibits the attachment on HA of *Prevoteiia ni- grescens* by binding to both HA and bacteria (49). Apo- Lf reduces the initial attaohmenfiaf the commensal *Streptococcus gordonii* by iron séquestration, but not that of pe- riodontopathogens *Fusobacterium nucleatum* and *P. gin-givaiis.* Interesting the ˙initial attachment of mixed populations qf *S. cjbrdonttR nucleatum* and S. *gordoniilP. gin- givaiis* is significantly reduced in the presence respect to that observed in the absence of Lf (50). in other studies, Lf has been shown to inhibit the adhesion of *A. actino- mycetemcon ˙*ans* and *P. intermedia* to reconstituted basement membrane, through ionic binding, and *P. intermedia* to bacterial adhesins by a specific binding of Lf (51).

The different nature of abiotic surfaces, the varying microbial adhesion mechanisms and the different in vitro experimental conditions could explain the different results obtained for inhibition of bacterial adhesion by apo- or iron- saturated Lf, which in some cases requires only ionic binding to biomaterials, and in others specific binding to bacterial structures, or both.

Ceil surfaces

The ability of microbes to adhere, colonize and form biofilm on host cells is a crucial step for the development and persistence of infections.

A large number of Gram-positive and Gram-negative bacteria possess specific adhesins that mediate the adhesion process on epithelial host ceils. Lf and Lfcin are ail able to bind to bacterial Gram-negative and Gram-positive surfaces, as well as to host cells, by binding to glycosami- noglycans (GAGs) (52) and in particular to heparan sulphate (HS) (53) and the inhibition of bacterial adhesion seems generally to be mediated by Lf binding to both bacterial and host cell surfaces (1).

Evidences of anti-adhesive function of Lf against oral pe-riodontopathogens was shown by Alugupally (1997) (51) who demonstrated that the Lf-dependent inhibition of the adhesion of A. *actinomycetemcomitans, P. intermedia* and *P. nigrescens* to fibroblasts can involve binding of lactoferrin to both the bacteria and host cells.

Recently, the Lf influence on *S. mutans* adhesion has been tested in vitro on epithelial cells. Lf at physiological con-centration (20 pig/ml) is able to significantly decrease the adhesion efficiency of *S. mutans* to epithelial cells (Fig. 2).

Inhibition of microbial invasion of host cells by lactoferrin

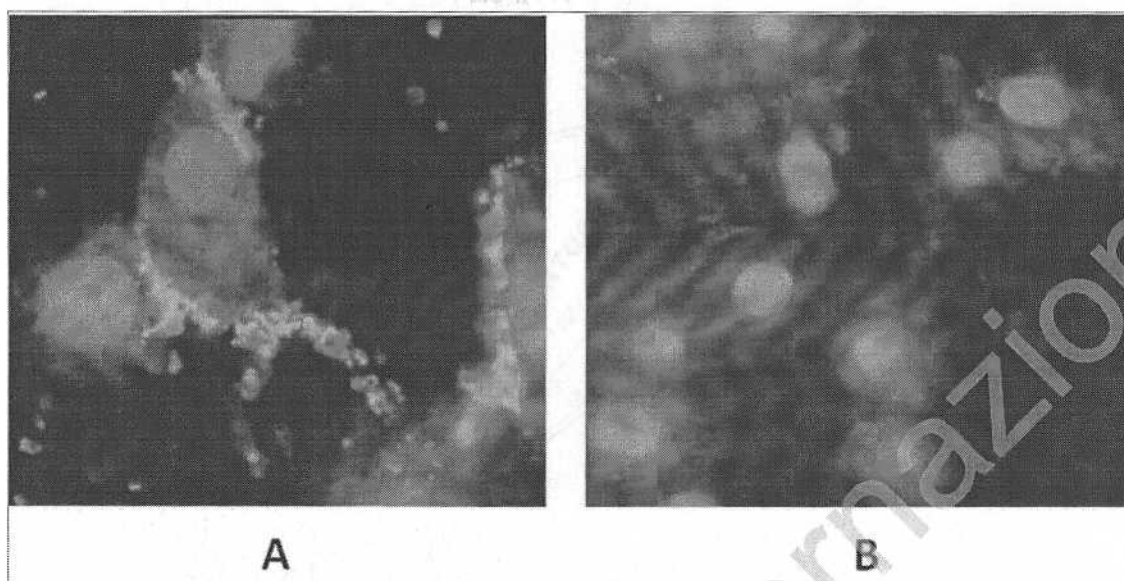Some mucosal pathogenic bacteria are capable not oniy

Figure 2 - Bovine lactoferrin in inhibiting adhesion efficiency of *Streptococcus mutans* on epithelial cells.
Legend: Epifluorescence optical microscopy images of epithelial cells infected with *Streptococcus mutans,* stained with Ba-cLight+LIVE/DEAD viability probe, in the absence (Panel A) or in the presence (Panel B) of bovine lactoferrin (2G pg/ml).

of adhering, but also of entering into non-professional phagocytes, such as epithelial cells. Inside host cells, bacteria are in a protective niche in which they can replicate and persist, thus avoiding host defences. Virulence determinants, such as surface proteins able to bind host cells, play a key role in the entry process inside the host cells. Lf has been shown to inhibit the entry of facultative intracellular bacteria, both Gram-negative and Gram-positive (1). Recently, the capability of *S. mutans* to enter inside gingival fibroblast cells has been reported (54). Preliminary experiments show the anti-invasive activity of Lf against S. *mutans* infecting gingival fibroblasts.

Anti-inflammatory activity of lactoferrin in oral diseases
Understanding the rote of microbial communities in human health is emerging as a fundamental and fascinating microbiological challenge, in the battle against infectious diseases, finally there is the awareness that the discovery of novel connections between infection, inflammation and human diseases is a pivotal tool in the research of novel antibacterial drugs.
The increasingly frequent threats of bacterial resistance to antibiotics reinforce the necessity to understand the me

chanisms that underlie pathogenicity and the interactions between pathogenic and non-pathogenic microbes coexisting on humans. These motivations are especially pertinent in the case of the human oral flora, which comprises at least 400 to 700 different bacterial species (55). Oral microorganisms constitute a complex and dynamical community, responsible for two important oral infectious diseases affecting virtually all humans: gingival and periodontal diseases. Differently from gingivitis, confined to gingival mucosa, periodontitis involves periodontium degeneration, alveolar bone resorption and gingival epithelium migration along tooth surface, and the resulting periodontal pockets.
It is well know that periodontal disease is associated to an inflammatory process that occurs in the tissues surrounding the teeth in response to the accumulation of subgingival bacterial plaque, mainly constituted by anaerobic Gram-negative facultative intracellular pathogens and their LPS, one of the factors responsible for the inflammatory reaction (56, 57, 58). Differently from commensal bacteria, intracellular bacteria induce the over-expression of several pro-inflammatory cytokines (59, 60, 61). In particular, increased levels of pro-inflammatory cytokines have

Table 2 - Synthesis of pro-inflammatory cytokines by gingival fibroblasts uninfected or infected with *Prevotella intermedia* in the absence or in the presence of bovine iactoferrin.

	Uninfected ceils		Infected cells	
	None	bLf	None	bLf
!L-1ß (ng/ml)	1.050±150	1.000 ±97	4.800±421	900±180
IL-6 (ng/ml)	90+21	90±23	930±105	83±38
IL-8 (pg/ml)	2.100±241	2.150±210	9.450±750	5.800±550
TNF-a (pg/ml)	22+14	22±12	120±65	18±8

in the absence or in the presence of 20 pg/ml of bLf. After infection, the supernatants from uninfected and infected cells with or without bLf were collected and IL-1 , IL- 6, IL-8 and TNF-o: concentrations were determined using standard enzyme-linked immunosorbent assays (ELISA) (Table 2).

The results clearly demonstrated that bLf added to uninfected monolayers did not modify cytokine synthesis, while it significantly decreased the synthesis of IL-1 , IL-6, IL- 8 and TNF-a by gingival fibroblasts Infected with *P. intermedia*.

These *in vitro* results have encouraged us to carry out an observational preclinical study on volunteers suffering from mild chronic periodontitis. The calibration was performed before the study with 5 volunteer subjects among the re-searchers involved in the study of bLf functions. Then other volunteers suffering from mild chronic periodontitis among patients were enrolled at a private practice. Pregnant women as well as the subjects with other concomitant diseases were excluded from this study as well as smokers, subjects with ascertained allergic reactions to cow's milk, and subjects receiving antibiotic treatment within the previous 3 months.

As a result, 13 subjects (7 female and 6 men, age range 42-63 years) suffering from mild chronic periodontitis were enrolled in this observational preclinical trial after the understanding and written consent. The trial was conducted

according to the World Medical Association Declaration of Helsinki.

At each visit, GCFs to be analyzed were collected from two teeth affected by periodontitis/subject. Ail subjects were cli-nically evaluated in the following periodontal measurements: number of teeth present, plaque index (Pll) (80), gingiva! index (G I) (81), probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) (82).Two teeth affected by periodontitis were examined per subject. Pll, GI, PD, CAL and BOP (%) were measured at four sites around each tooth.

All enrolled subjects were directed to topically place 100 mg of lyophilized bLf two times a day for 4 weeks lifter meal and correct oral hygiene as well as after extensive washings until the arrest of the putative gum bleeding. In particular, 100 mg of lyophilized bLf, contained in a pocket, were deposited in an little container in order to be adsorbed on soft bristles of the appropriate toothbrush and applied through a soft brush on the gums, particularly on the bleeding sites.

Periodontal clinical parameters consisting in PPD, GI, Pli, BOP (%) and cytokine concentrations in GCFs were assayed in all subjects before (baseline) and after 1,2 and 4 weeks of therapy. The parameters at the baseline and after 4 weeks of bLf topical treatment are reported as cumulated mean values because the total number of subjects in each grotip (seven women and six men) is very low (Ta-
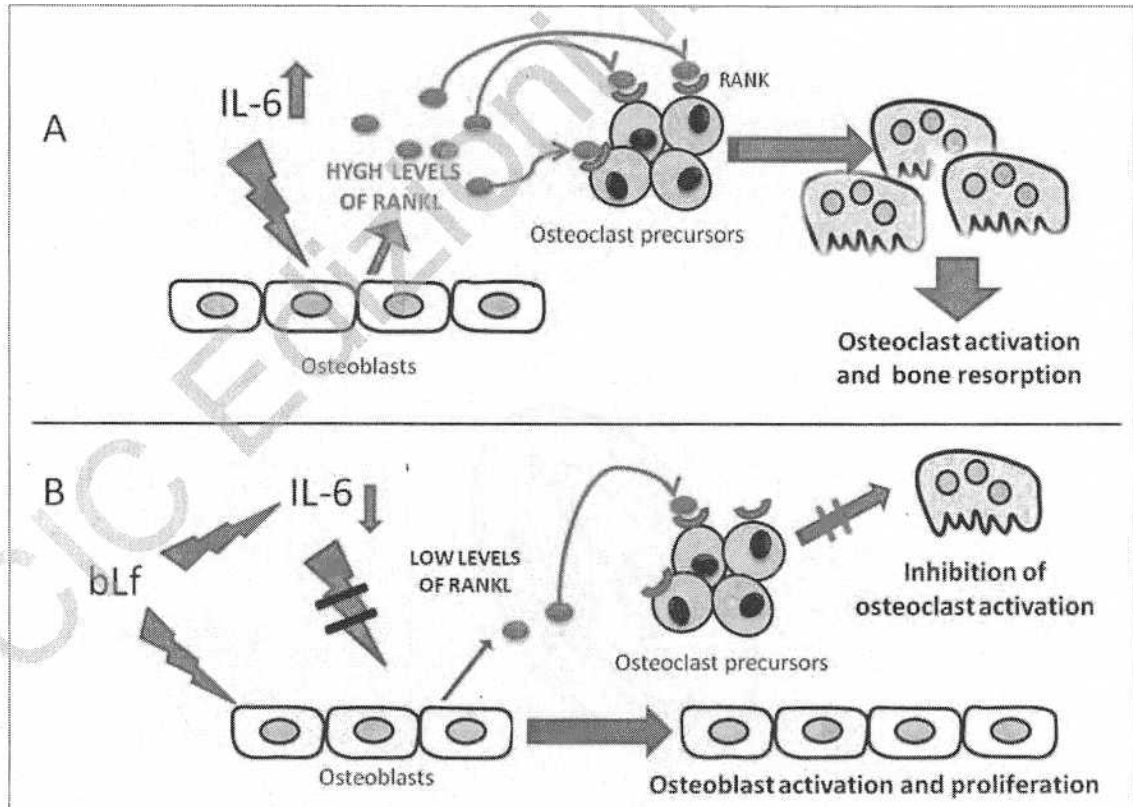

Figure 3 - Putative lactoferrin mechanism in inhibiting bone resorption. High levels of IL-6 induce bone resorption through the osteoclast activation induced by the osteoblast-mediated production of RANKL (Panel A). The topical administration of bLf by decreasing IL- 6 levels and probably RANKL may induce both the inhibition of osteoclast activation and the activation and proliferation of osteoblast (Panel B). Legend: IL-6: interleukin-6; bLf: bovine lactoferrin; RANK: receptor activator of nuclear factor NFkB; RANKL: RANK ligand.

ble 3). Therefore, the data summarized in Table 3 correspond to the mean values deriving from a total of 13 subjects and 26 teeth.

From the data reported in Table 3, it can be observed that bLf topical administration decreased the concentrations of pro-inflammatory cytokines in GCF, thus decreasing oedema and inflammation. Moreover, after four weeks of topical bLf therapy, the absence of bleeding, the reduction of pocket depth, gingival and plaque index as well as an improvement of clinical attachment level were observed. Interestingly, no adverse events were reported in the subjects receiving topical administration of bLf.

In conclusion, Lf is a component of saliva and an important defence factor against bacterial injuries (1) including those related to *S. mutans* (54) and to other representative perlodontopathic bacteria as *A. actinomycetemco- mitans, P gingivalis,* and *P. intermedia* (83). Periodonto- pathic bacteria reside as a biofilm in supragingival and subgingival plaque and Lf is able to reduce their biofilm development (84). Even if in a small-scale clinical study, oral administration of bLf has been found to reduce *R gingivalis* and *P. intermedia* in the subgingival plaque of chronic periodontitis patients thus suggesting that the inhibitory effects of Lf on biofilm development of periodontopathic bacteria can have a potential use in prevention, treatment of periodontal diseases (83).

However, it cannot be undervalued the importance of the inflammatory process associated with periodontal disease. Consequently, an appropriate regulation of pro-inflammatory cytokine synthesis might be useful in reducing periodontal tissue destruction. Recently, among pro-inflammatory cytokines, the multifunctional IL-6 is emerging as an important factor able to modulate bone, iron and inflammatory homeostasis (79, 67).

At present, there is no ideal therapeutic approach to Btire periodontitis and achieve optima! periodontal tissue re-generation.

Here, firstly we report that the topical administration of bLf is able to reduced pro-inflammatory cytokines in GCF thus contemporary decreasing oedema end inflammation, bleeding, pocket depth, gingival and plaque index.This potent effect of bLf topically administered results in an important improvement of clinical attachment level, probably due to the ability of bLf to decrease the synthesis of IL-6 and RANKL thus resulting in the inhibition of bone resorption (Figure 3).

Even if other clinical trials are required, these results provide strong evidence for a role of bLf in curing periodontitis, thus extending the therapeutic potential of this mul- : Afunctional natural protein.

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