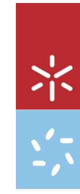
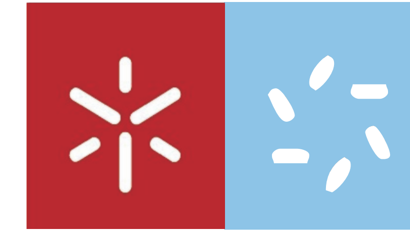


Molecular Mechanism Underlying the Anti-Tumoral Activity of Lactoferrin

Cátia Pereira

Uminho | 2014



Universidade do Minho
Escola de Ciências

Cátia Sofia dos Santos Pereira

Molecular Mechanism Underlying the Anti-Tumoral Activity of Lactoferrin

Dezembro de 2014

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Escola de Ciências

Cátia Sofia dos Santos Pereira

Molecular Mechanism Underlying the Anti-Tumoral Activity of Lactoferrin

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Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real
Professora Doutora Lígia Raquel Marona Rodrigues

Dezembro de 2014

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MOLECULAR MECHANISM UNDERLYING THE ANTI-TUMORAL ACTIVITY OF LACTOFERRIN

ABSTRACT

Lactoferrin (Lf) is an iron-binding glycoprotein found in many biological fluids, being particularly abundant in milk. Originally viewed as a milk protein involved in the regulation of iron homeostasis, Lf is now considered a multifunctional protein to which more and more physiological roles have been attributed. These include anti-tumoral, anti-microbial, immunomodulatory, among other properties. Regarding its anti-tumoral activity, Lf has been reported to be effective against a variety of human cancers both by *in vitro* an *in vivo* studies. Its well-tolerability has also been attested in some human clinical trials. Therefore, Lf administration is an emerging strategy in cancer therapeutics that is expected to become widespread. In this way, the elucidation of molecular mechanisms underlying Lf cytotoxicity to cancer cells has become an important research field. Although some cellular mechanisms have already been proposed such as apoptosis induction, cell cycle arrest, angiogenesis and metastasis inhibition, the true molecular mechanism remains elusive. In the present work, we aimed to unveil the mechanism of action and the cellular target(s) of Lf on cancer cells. For that purpose, three breast cell lines with different genetic backgrounds were used, namely an invasive, a non-invasive and a non-tumorigenic. In this study we focus on bovine Lf (bLf) as it has been considered a safe nutraceutical for various applications. Results showed that these cells exhibit different susceptibilities to bLf, which prompted us to implement several biochemical and analytical approaches, including flow cytometry, fluorescence microscopy and spectrofluorimetry in order to dissect the bLf's target(s) and molecular mechanism operating in the sensitive cells. Results obtained throughout this work allowed us to identify a novel Lf activity and to propose a molecular mechanism regarding its anti-tumoral activity, which may have important implications on cancer therapeutics, particularly on the therapy of highly metastatic tumors.

RESUMO

A lactoferrina (Lf) é uma glicoproteína de ligação ao ferro encontrada em diversos fluídos biológicos, sendo particularmente abundante no leite. Originalmente descrita como uma proteína do leite envolvida na regulação da homeostasia do ferro, a Lf é atualmente considerada uma proteína multifuncional à qual têm sido atribuídas diversas funções fisiológicas. Estas incluem propriedades anti-tumorais, anti-microbianas, imunomoduladoras, entre outras. Relativamente à sua atividade anti-tumoral, a Lf foi descrita como eficaz contra uma variedade de cancros humanos em estudos realizados *in vitro* e *in vivo*. A sua boa tolerabilidade foi também demonstrada em alguns ensaios clínicos em humanos. Portanto, a administração de Lf constitui uma estratégia com elevado potencial na terapêutica do cancro, sendo expectável que se torne generalizada. Desta forma, a elucidação dos mecanismos moleculares subjacentes às suas propriedades anti-tumorais tornou-se um tópico importante de investigação. Embora tenham sido já propostos alguns mecanismos de ação, tais como a indução de apoptose, a paragem do ciclo celular, a inibição da angiogénese e da metastização, o mecanismo molecular preciso pelo qual esta proteína atua permanece desconhecido. O objetivo do presente trabalho consistiu em estudar o mecanismo de ação e os potenciais alvo(s) celular(es) da Lf nas células cancerígenas. Para esse efeito, foram utilizadas três linhas celulares de mama com diferentes características genéticas, nomeadamente, uma invasiva, uma não-invasiva e uma não-tumorigénica. Neste estudo, foi utilizada a Lf bovina (bLF) por ser considerada um nutracêutico seguro para várias aplicações. Os resultados mostraram que as linhas celulares apresentam diferentes sensibilidades à bLF, o que motivou a aplicação de diversas abordagens bioquímicas e analíticas, incluindo citometria de fluxo, microscopia de fluorescência e espectrofluorimetria, para dissecar o(s) alvo(s) e o mecanismo molecular de ação da bLf nas células sensíveis. Os resultados obtidos ao longo deste trabalho permitiram identificar uma nova atividade da Lf e propor um mecanismo molecular envolvido na sua atividade anti-tumoral, com implicações potencialmente importantes na terapêutica de cancro, particularmente na terapia de tumores altamente metastáticos.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACMA	9-**A**mino-6-**C**hloro-2-**M**ethoxy**A**cridine
ANOVA	**AN**alysis **O**f **VA**riance
ATCC	**A**merican **T**ype **C**ulture **C**ollection
ATP	**A**denosine **T**ri**P**hosphate
BafA1	**Baf**ilomycin **A1**
BCECF-AM	2',7'-**B**is-(2-**C**arboxy**E**thyl)-5-(and-6)-**C**arboxy**F**luorescein - **A**cetoxy**M**ethyl Ester
bLf	**b**ovine **L**acto**f**errin
BSA	**B**ovine **S**erum **A**lbumin
CAs	**C**arbonic **A**nhydrase**s**
Caspase	**C**ystein-dependent **a**spartate **s**pecific **p**rote**ase**
Cdk	**C**yclin-**d**ependent **k**inase
ConcA	**Conc**anamycin **A**
DMEM	**D**ulbecco's **M**odified **E**agle's **M**edium
DNA	**D**eoxyribo**N**ucleic **A**cid
DRMs	**D**etergent-**R**esistant **M**embrane**s**
ECM	**E**xtra**C**ellular **M**atrix
EFSA-NDA	**E**uropean **F**ood **S**afety **A**uthority – Panel on **D**ietetic Products, **N**utrition and **A**llergies
F	**F**luorescence
FITC	**F**luorescein **I**so**T**hio**C**yanate
GAPDH	**G**lycer**A**ldehyde-3-**P**hosphate **D**e**H**ydrogenase
HBSS	**H**ank’s **B**alanced **S**alt **S**olution
HEPES	4-(2-**H**ydroxy**E**thyl)-1-**P**iperazine**E**thane**S**ulfonic acid
hLf	**h**uman **L**acto**f**errin
IFN	**I**nter**F**ero**N**
IFP	**I**nterstitial **F**luid **P**ressure
IgG	**I**mmuno**g**lobulin **G**

IL	**I**nter**L**eukin
K_m	Michaelis constant
Lf	**L**acto**f**errin
MCT	**M**ono**C**arboxylate **T**ransporter
MMP	**M**atrix **M**etallo**P**roteinase
MOPS	3-(N-**MO**rpholino)**P**ropane**S**ulfonic acid
MβCD	**M**ethyl-**β**-**C**iclo**D**extrin
NK	**N**atural **K**iller
PARP	**P**oly (**A**DP-**R**ibose) **P**olymerase
PBS	**P**hosphate **B**uffered **S**aline
PFA	**P**ara**F**orm**A**ldehyde
pHe	**e**xtracellular **pH**
Phenol Red	Phenolsulfonephthaleine
pHi	**i**ntracellular **pH**
Pi	**i**norganic **P**hosphate
PI	**P**ropidium **I**odide
pK_a	Dissociation constant
PPI	**P**roton **P**ump **I**nhibitor
PS	**P**hosphatidyl**S**erine
PVDF	**P**oly**V**inylidene **D**i**F**luoride
Rb	**R**etino**b**lastoma
RNA	**R**ibo**N**ucleic **A**cid
ROS	**R**eactive **O**xygen **S**pecies
rpm	**r**otations **p**er **m**inute
RT	**R**oom **T**emperature
S.E.M.	**S**tandard **E**rror of the **M**ean
SDS	**S**odium **D**odecyl **S**ulfate
siRNA	**s**mall **i**nterfering **RNA**
SRB	**S**ulfo**R**hodamine **B**
TCA	**T**ri**C**hloro**A**cetic acid
TME	**T**umor **M**icro**E**nvironment

t/yr	**t**ons per **y**ear
TUNEL	**T**erminal deoxynucleotidyl transferase-mediated d**U**TP **N**ick **E**nd **L**abeling
V-ATPase	**V**acuolar-type proton-translocating **ATPase**
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor

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Chapter I

INTRODUCTION

I.1. LACTOFERRIN: A MULTIFACETED IRON BINDING PROTEIN

Transferrins are a family of proteins that comprises iron-binding polypeptides of diverse phylogenetic groups that, by taking advantage of their ability to bind tightly two ferrin ions (Fe^{3+}), control the levels of iron in the biological fluids of vertebrates (Lambert et al., 2005). Two well-known representative members of this family are transferrin and lactoferrin. In the present work we will focus on lactoferrin.

Lactoferrin (Lf), also known as lactotransferrin, is an 80 kDa non hemic iron-binding glycoprotein of 700 amino acids (Ward et al., 2005; García-Montoya et al., 2012), first identified in 1939 in bovine milk - bLf (Soerensen and Soerensen, 1939) and isolated in 1960 from both human - hLf (Johansson, 1960) and bovine milk (Groves, 1960). Lf is a cell-secreted protein produced by the epithelial cells of mucosa, or alternatively released at high concentrations from the secondary granules of activated neutrophils upon inflammatory processes (Gifford et al., 2012). Hence, it is found in many biological fluids such as saliva, tears, bile, pancreatic, gastric and vaginal fluids, semen, urine, nasal and bronchial secretions, and at highest concentrations in milk and colostrum, making it the second most common protein in milk, after caseins (Alexander et al., 2012; González-Chávez et al., 2009). It is found in various mammalian species, including humans, cows, goats, horses, dogs, several rodents, and also in fishes, displaying high homology among some species (González-Chávez et al., 2009). Indeed, bLf has 69% sequence homology with hLf at the protein level and 77% sequence homology at the mRNA level (Liao et al., 2012), and has been demonstrated to have similar effects to hLf (Buccigrossi et al., 2007; Liao et al., 2012; El-Fakharany et al., 2013).

The three-dimensional structure of Lf, defined by X-ray crystallography, revealed a globular protein folded into two highly homologous iron-binding lobes (N and C) which are further divided into two domains (N1 and N2, C1 and C2) (Fig. I.1 A). Each of these lobes can reversibly bind one ferric ion (Fe^{3+}) in the deep cleft between the two domains, with the concomitant binding of a bicarbonate anion (Berlutti et al., 2011; Mizutani et al., 2012).

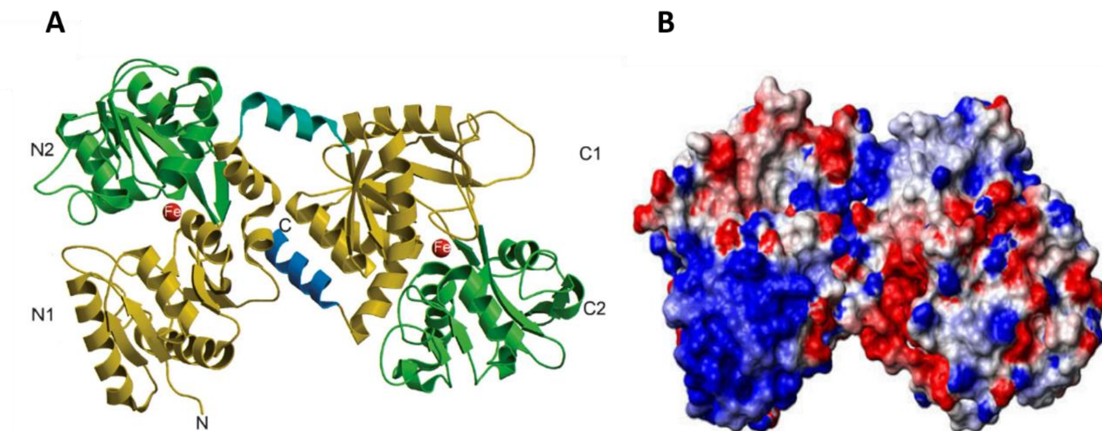


Figure I.1: Structure of lactoferrin. (A) Crystal structure of Lf illustrating its N1, N2, C1 and C2 domains. The α-helices that bind the two domains are shown in blue and bound iron in red. **(B)** Charge distribution plot on the surface of bLf with colors blue, white and red corresponding to net positive, neutral and negative charge, respectively, thus emphasising the highly cationic N-terminal portion of the protein (adapted from Jenssen and Hancock, 2009; Baker and Baker, 2012).

Because of its ability to reversibly bind Fe^{3+}, Lf can exist free of Fe^{3+} (apo-Lf) or associated with it (holo-Lf). Actually, it is secreted in the apo form and, in the human milk, native Lf is a combination of 10% holo-Lf and 90% apo-Lf isoforms (Buccigrossi et al., 2007). Depending on whether or not Lf is bound to iron, it has a different three-dimensional conformation. Apo-Lf has an open conformation, while holo-Lf is a closed molecule with greater resistance to proteolysis. Iron binding and release is thus associated with large-scale conformational changes in which the domains close over the ion or open to release it (Baker and Baker, 2012). Lf has a great iron-binding affinity, being able to preserve this metallic cation over a wide pH range, including extremely acidic pH. In fact, Lf has been proved to retain bound iron at a pH as low as ~3.5, and also to be more active at acidic pH (Day et al., 1992; Andrés and Fierro, 2010).

Because of its high positive charge (pI ~ 9), and particularly its highly cationic N-terminal region (Fig. I.1 B), Lf is capable of binding to other compounds, namely heparin, lipopolysaccharides, DNA (He and Furmanski, 1995; Berkel et al., 1997), glycosaminoglycans, as well as other metal ions such as Al^{3+}, Ga^{3+}, Mn^{3+}, Co^{3+}, Cu^{2+} and Zn^{2+}; or other anions like oxalates and carboxylates. However, its affinity for these other

compounds is much lower. Taking this into account, it is likely that Lf affects the metabolism and distribution of various substances, exhibiting very different biological functions (Baker and Baker, 2004; Adlerova et al., 2008).

Lf synthesis can be constitutive, hormone-dependent or occur at well-defined stages of cell differentiation. The constitutive synthesis takes place in the mucosal surfaces. Alternatively, Lf synthesis can be regulated by hormones in a tissue-specific manner. For example, in the mammary gland it is controlled by prolactin and in reproductive tissues by estrogens. Finally, Lf is synthetized by neutrophils during their differentiation process (reviewed by Adlerova et al., 2008; Legrand et al., 2008).

Another interesting feature about Lf is that it can also be found in the lysosomes where it exhibits a quite different behavior. Lf is localized at the lysosomes in hepatic cells upon its removal from the organism, which is carried out by the liver, as well as by phagocytic cells (Levay and Viljoen, 1995). Indeed, lysosomal Lf was initially found to activate procaspase-3 leading to apoptosis and, more recently, an apoptotic cascade mediated by lysosomal Lf was described, involving its release from the lysosome to the cytosol. In addition, it was documented the protective effect of tea epigallo-catechin gallate which suppresses the release of Lf from the lysosome. However, the mechanism by which Lf is released is still unknown (Katunuma et al., 2004, 2006). Furthermore, it was reported the existence of a lysosome-related pathway for caspase-3 activation upon benzo[a]pyrene exposure in hepatic epithelial cells, which is dependent on both iron content and Lf presence in lysosomes (Gorria et al., 2008).

I.1.1. Biological Functions of Lactoferrin

Over the past 50 years, an overwhelming number of potential biological activities have been proposed for Lf, being it currently defined as a multifunctional protein, in part due to its extensive distribution in many tissues (Brock, 2012). Indeed, it is involved in many physiological functions, some of them associated with its iron binding ability (Baker and Baker, 2004). Generally, Lf plays a role in the regulation of iron absorption in the bowel and in the immune response; it has anti-oxidant, anti-tumoral, immunomodulatory and anti-inflammatory properties; and exerts protection against microbial infection, which is the most widely studied function (González-Chávez et al.,

2009; Yen et al., 2011). Figure I.2 summarizes the multitude of activities that have been proposed for Lf.

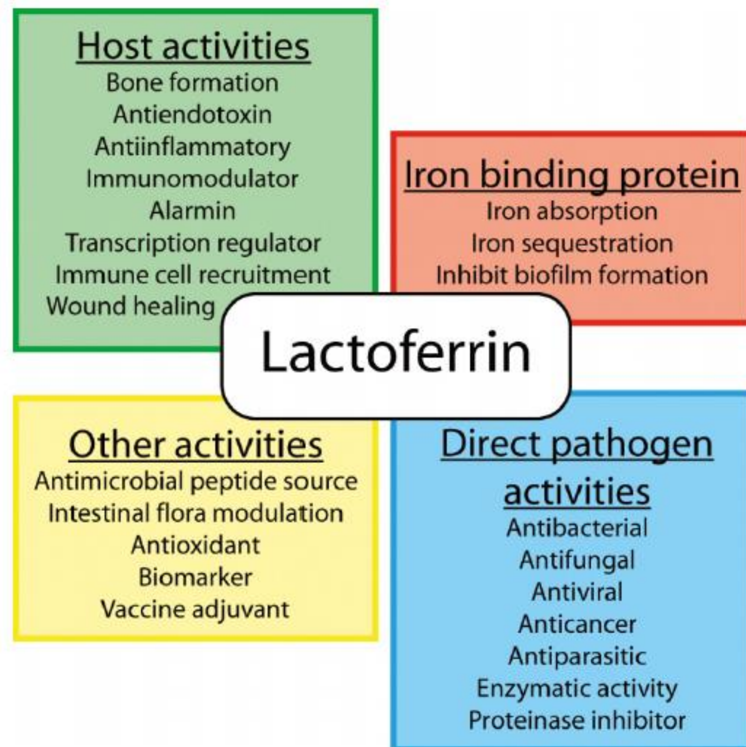


Figure I.2: Multiple activities described for Lf (Vogel, 2012).

Concerning the anti-microbial properties of Lf, it is now well recognized that this protein plays a direct anti-microbial role in secretions and at the surface of epithelia. It has been documented that Lf exhibits strong anti-microbial activity against a broad spectrum of bacteria (Gram + and Gram -), fungi, yeasts, protozoa, viruses and parasites (reviewed by Legrand et al., 2008; González-Chávez et al., 2009; García-Montoya et al., 2012). The reasons why Lf exerts such a plethora of anti-microbial activities have been investigated and some mechanisms have been put forward, including iron sequestering (Zarember et al., 2007), direct interaction with the cell surfaces (Xu et al., 1999), impairment of adhesion to host cells (Diarra et al., 2003), inhibition of biofilm formation (Dashper et al., 2012), stimulation of the host immune system (Welsh et al., 2011), among others.

Moreover, both immunomodulatory and anti-inflammatory properties have been attributed to Lf. In fact, this protein is now considered a key component in the host

first line of defence having the ability to modulate the overall immune response (Puddu et al., 2009). This insight into the biological role of Lf arose when researchers found that this protein was abundant in neutrophils (Masson et al., 1969). In fact, Lf is a major component of the secondary granules of neutrophils, which is released through degranulation upon neutrophil activation, ultimately resulting in increased levels of Lf. Then, by interacting and promoting the recruitment of leukocytes and activation of dendritic cells, as well as by modulating the expression of soluble mediators like cytokines and chemokines, it controls the excessive inflammation and enhances the immune response (Cumberbatch et al., 2003; de la Rosa et al., 2008; Yamano et al., 2010).

Another fundamental feature of Lf is its ability to function as an enzyme. Besides the protease activity (Hendrixson et al., 2003), it was found that some subfractions of Lf obtained by chromatography possess five other enzymatic activities, namely DNase, RNase, ATPase, phosphatase, and amylase. This variety of activities can be attributed to variations in the protein characteristics like degree of glycosylation and oligomerization, and tertiary structure (Kanyshkova et al., 2003). The elucidation of the Lf enzymatic properties might help to understand its multi-activities as the nuclease activity is suggested to contribute to its anti-microbial properties.

The multiple functions of Lf are in part mediated by its specific receptors at the surface of target cells. Far more research is warranted in this area but some receptors have already been suggested in bacteria (Ling and Schryvers, 2006) and in mammals. Effectively, Lf receptors can be found in various mammalian tissues such as liver, monocytes, lymphocytes and bone (Suzuki et al., 2005), nonetheless the most studied is intelectin 1, the lactoferrin receptor in the human intestinal mucosa (Akiyama et al., 2013). Lf has been demonstrated to be internalized in some cell types (Lopez et al., 2008; Akiyama et al., 2013), however there are also some cells unable to internalize Lf or able to internalize only a small amount. This is the case of some cancer cells such as MCF-7 (Baumrucker et al., 2006), T-47D and MDA-MB-231 breast cancer cell lines (Zhang et al., 2014a).

In this work, we will explore the anti-tumoral activity of the lactoferrin.

I.1.1.1. Anti-Tumoral Activity of Lactoferrin

Cancer is a leading cause of death worldwide and accounted for 8.2 million deaths (22% of all deaths) in 2012 (WHO, 2014). It has been defined as a disease consisting of transformed cells acquiring cell autonomous hyper-proliferative, invasive and limitless survival capacities that then undergo a transition to expanding masses with metastatic propensity (van Kempen et al., 2003; Lorusso and Rüegg, 2008).

A growing number of reports suggest the Lf's benefits against cancer and studies with various cancer cell lines and animal models have been reported, all showing the favorable effects of Lf (Gibbons et al., 2011). This topic has been thoroughly reviewed by Rodrigues et al., 2009; Tsuda et al., 2010; Vogel, 2012, among many others. One of the initial findings suggesting the anti-tumoral activity of Lf was published in 1995. In this study, it was established that the whey fraction of bovine milk could significantly inhibit the development of colon tumors in rats (McIntosh et al., 1995). Then, new understanding on the biological role of Lf in cancer emerged when its expression was found to be downregulated in many types of cancer. In this context, Lf was suggested to negatively regulate the tumor progression, thus acting as a tumor suppressor (Zhou et al., 2008; Deng et al., 2013). Nowadays, there are numerous studies reporting the anti-tumoral activity of Lf in several types of cancer such as lung (Tung et al., 2013), colon (Fujita et al., 2004), breast (Duarte et al., 2011), stomach (Xu et al., 2010), cervix (Shi and Li, 2014), leukemia (Lee et al., 2009), head and neck (Wolf et al., 2007), bladder (Masuda et al., 2000) and melanoma (Roseanu et al., 2010).

All those years of research allowed the identification of some mechanisms that might underlie the Lf anti-tumoral activity (Fig. I.3). One of them is dependent on Lf iron-binding ability (Fig. I.3 D). The carcinogenicity of iron compounds has been demonstrated in various experiments and free iron has been suggested to act as a mutagenic promoter by inducing oxidative damage to nucleic acids (reviewed by Toyokuni, 2009). Since Lf can bind iron locally in tissues, it may reduce the risk of oxidant-induced carcinogenesis (Rodrigues et al., 2009).

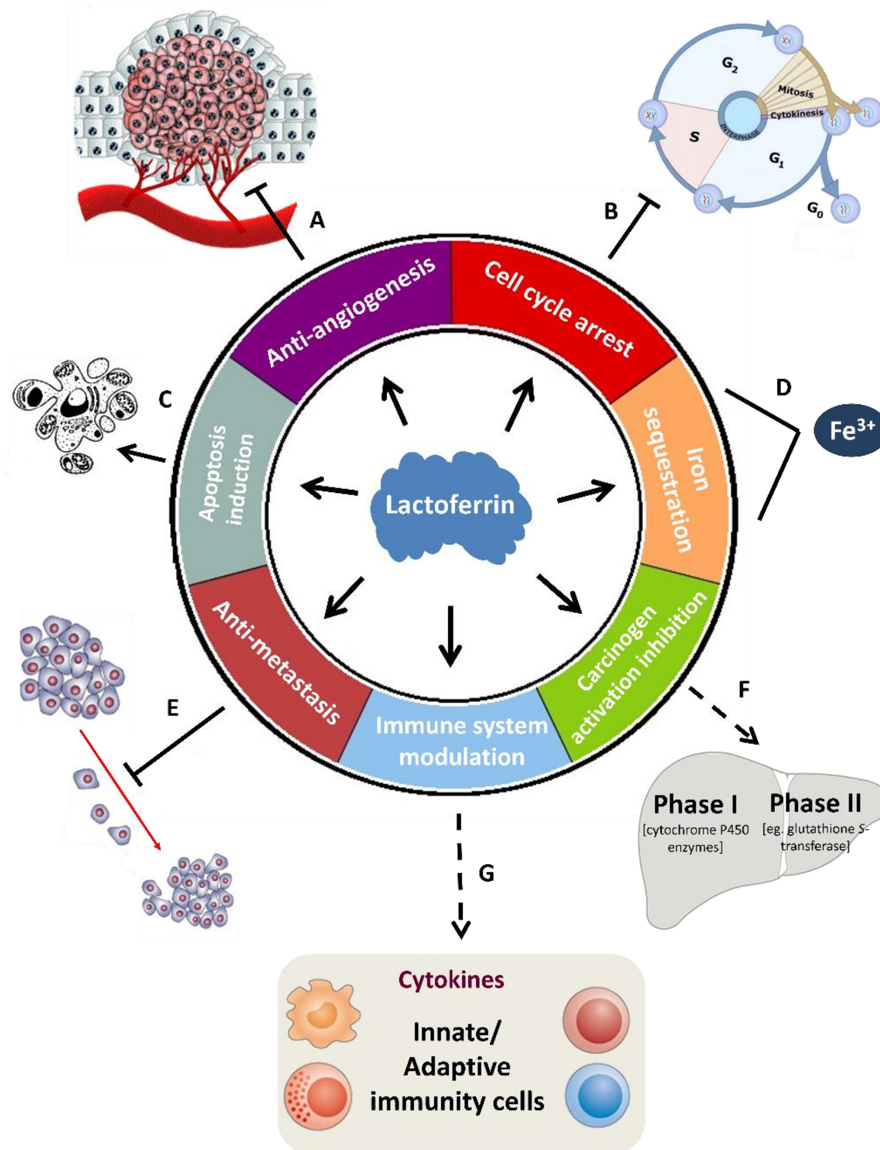


Figure I.3: Proposed mechanisms for the anti-tumoral activity of lactoferrin.

Legend: ⟶ Induction ⊣ Inhibition - -> Modulation > Sequestration (see text for references).

On the other hand, an immunomodulatory action for Lf in preventing carcinogenesis and impeding tumor progression has also been proposed (Fig. I.3 G). It has been reported that Lf has the ability to modulate the production of cytokines in cancer cells and to stimulate the production and/or activation of several immune cells (Ward et al., 2005; Wolf et al., 2007). For instance, Lf administration seems to increase the production of the pro-inflammatory cytokine interleukin-18 (IL-18) in the intestinal tract and to systemically activate the natural killer (NK) cells and $CD8^+$ T lymphocytes in the circulation which exhibited marked cytotoxicity against murine colon carcinoma

26Lu (Co26Lu) cells and decreased the formation of lung metastatic colonies *in vitro* (Wang et al., 2000). Generally, Lf enhances the production of many cytokines including IFNα, β and γ, IL-18, IL-12 and IL-7, and it modulates the activation of cells involved either in innate or adaptive immunity, most particularly dendritic cells, macrophages, neutrophils, NK cells and various T cell subsets (reviewed by Legrand and Mazurier, 2010; Tsuda et al., 2010).

Lf anti-tumoral activity also relies on its ability to trigger apoptosis (Fig. I.3 C). Apoptosis is a cellular suicide process in which a programmed sequence of events culminates in the death of cells without the release of harmful substances into the surrounding area (Meier and Vousden, 2007). This process has been extensively studied in different models and various apoptotic markers induced by Lf have been described (Table I.1). Furthermore, the levels of some apoptosis-related proteins were found to be altered in Lf-treated tumor cells. In HeLa cervical carcinoma cells, Lf was shown to transactivate the tumor suppressor protein p53, known to induce apoptotic cell death, and its target genes *mdm2* and *p21* (Oh et al., 2004). In human stomach and breast cancer cell lines, Lf was found to downregulate the levels of the anti-apoptotic protein Bcl-2 (Xu et al., 2010; Zhang et al., 2014b). In mice bearing EMT6 breast cancer, a recombinant form of Lf induced apoptosis by decreasing the expression of Bcl-2 and increasing the expression of the pro-apoptotic Bax and the executioner caspase-3 at both mRNA and protein level (Wang et al., 2011).

Furthermore, accumulating evidence suggest that cell cycle arrest might play a critical role in Lf anti-tumoral activity (Fig. I.3 B). Several authors documented the Lf-induced cell cycle arrest, which occurs predominantly at the G1 phase (Wolf et al., 2007; Wang et al., 2011). Specifically, in head and neck cancer cell lines, Lf caused growth arrest at the G1 to S transition of the cell cycle by downregulating the G1 cyclin-dependent kinases (Cdk), and increasing the expression of p21 and p27, which are known inhibitors of the cell cycle (Xiao et al., 2004). A similar effect was found in Lf-treated breast cancer cells (Damiens et al., 1999). The retinoblastoma protein (Rb), a key tumor suppressor gene involved in the inhibition of cell cycle progression, was also affected by Lf in these and other cell lines, since its phosphorylated/inactive form was downregulated (Damiens et al., 1999; Xiao et al., 2004; Son et al., 2006). Moreover, it was also reported that the inhibitory effect of Lf in the cell cycle progression was cell

type-dependent, since in MDA-MB-231 breast cancer cells the arrest was observed at G2 phase, whereas in MCF-7 cells there was an arrest at G1 phase with low doses of Lf and at G2 phase with higher doses (Zhang et al., 2014a). In summary, the inhibition of cell proliferation, cell cycle arrest and modulation of the cell cycle regulatory proteins, together with the apoptosis induction are some of the main mechanisms described to explain the Lf anti-tumoral activity.

Table I.1: Apoptotic markers and respective techniques used to monitor the induction of apoptosis by lactoferrin. PS: phosphatidylserine; FITC: fluorescein isothiocyanate; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; PARP: poly (ADP-ribose) polymerase; PI: propidium iodide.

Models / Cell lines	Apoptotic Markers	Technique	Reference
MCF-7 human breast cancer cells	- PS exposure monitored by annexin V-FITC - Nuclear chromatin condensation - Mitochondrial depolarisation	Flow cytometry Fluorescence Microscopy	Wang et al., 2012a; Zhang et al., 2014b
Hs578T and **T47D** human breast cancer cells	- Caspase activation using the caspase-Glo 3/7 reagent	Luminescence recording	Duarte et al., 2011
Jurkat leukemia T cell line	- PS exposure monitored by annexin V-FITC - Detection of sub-G0/G1 peak	Flow cytometry	Lee et al., 2009
Colon tumors of rats injected subcutaneously with azoxymethane	- DNA fragmentation - Detection of caspase-8, caspase-3, Fas and PARP	TUNEL staining Western blot analysis	Fujita et al., 2004
SGC-7901 human stomach cancer cell line	- PS exposure monitored by annexin V-PI	Flow cytometry	Xu et al., 2010
Mice bearing **EMT6** breast cancer	- DNA fragmentation - Detection of sub-G0/G1 peak	TUNEL staining Flow cytometry	Wang et al., 2011

The anti-tumoral activity of Lf also stems from its anti-metastatic potential (Fig. I.3 E). In 1994, Bezault and co-workers demonstrated the inhibitory capacity of this protein in reducing the lung colonization derived from metastatic melanoma cells, in

mice (Bezault et al., 1994). Lung metastatic colony formation was also inhibited by Lf in mice bearing a highly metastatic colon carcinoma 26Lu (Iigo et al., 1999). Additionally, Lf was shown to inhibit lung and liver metastases derived from the metastatic L5178Y-ML25 lymphoma cells (Yoo et al., 1997). The protective role of Lf against metastasis has generally been attributed to enhanced immunity by increased production of IL-18 and consequent activation of NK and T cells (Iigo et al., 2004).

In addition, it was found that bLf possesses anti-angiogenic properties as it was capable of inhibiting vascular endothelial growth factor (VEGF)-induced angiogenesis in a rat model (Fig. I.3 A). Since tumor growth is angiogenesis-dependent, the suppression of new blood vessel growth might be implicated in the Lf anti-tumoral effects (Norrby et al., 2001). Shimamura and co-workers showed that this inhibition of angiogenesis is dose-dependent (Shimamura et al., 2004). More recently, it was demonstrated that bLf decreases the expression of VEGF mRNA and protein in a lung cancer cell line and inhibits the *in vivo* formation of lung cancer dependent of VEGF overexpression (Tung et al., 2013). Curiously, in what concerns angiogenesis, human and bovine Lf exert opposite effects. bLf inhibits angiogenesis while hLf was reported to have a specific pro-angiogenic effect in VEGF-A-mediated angiogenesis, which might be explained by differences in their molecular features (Norrby, 2004).

Another mechanism by which Lf is capable of exerting anti-tumor activities was demonstrated in models of chemically induced carcinogenesis (Fig. I.3 F). This process encompasses two stages, namely initiation and post-initiation. The initiation stage requires the activity of enzymes belonging to the liver detoxication metabolism, namely phase I enzymes like cytochrome P450 species. These enzymes activate the carcinogens leading to DNA damage in the target organs. This activation is blocked by liver phase II enzymes responsible for detoxication and excretion. The compounds that supress the activation of phase I enzymes are called “blocking agents”, whilst those capable of inhibiting the post-initiation phase by supressing the proliferation of pre-malignant cells are defined as “supressing agents”. Authors using different rat and hamster models of chemical carcinogenesis, found that orally administered bLf significantly inhibited colon, esophagus, lung, bladder and buccal pouch carcinogenesis. The study of this inhibitory effect provided clear evidence that bLf acts as a blocking and supressing agent by

inhibiting phase I enzymes, enhancing the phase II enzymes, or by preventing the proliferation of pre-malignant cells (Tsuda et al., 2002; Mohan et al., 2006).

Regarding Lf iron saturation status, several studies have encompassed the anti-tumoral activities of apo- (iron-free) and holo-Lf (iron-saturated). The results are controversial depending on the context, but holo-Lf has been shown to be more effective against cancer. In fact, Kanwar et al., 2008 conducted a fascinating work in mice bearing EL-4 lymphomas, Lewis lung carcinoma or B16 melanoma tumours, in which it was proved that mice fed with holo-Lf prior to chemotherapy completely rejected their tumors, whereas feeding with other forms of Lf containing a lesser iron-saturation degree (apo-Lf, natural-Lf and 50% iron-saturated Lf) did not. Notably, holo-Lf treatment restored red and white blood cell numbers decreased by chemotherapy. Holo-Lf was also demonstrated to augment chemotherapeutic effects of tamoxifen in a mouse model representing metastatic 4T1 breast cancer (Sun et al., 2012).

Finally, high-throughput approaches concerning the anti-tumor role of Lf have already been carried out. Importantly, a proteomic profiling of human MDA-MB-231 breast cancer cell line exposed to hLf was performed, and the classification of the proteins up-regulated in the presence of hLf showed that the majority was involved in the maintenance of cellular homeostasis with proteins involved in cell signalling, cell cycle and apoptosis (Hoedt et al., 2014), which is in agreement with the proposed mechanisms for the Lf anti-tumoral activity. Despite the fact that of all these data gathered by several research groups point to a clear anti-tumoral role for Lf, the mechanisms by which it exerts these effects are not fully understood (Rodrigues et al., 2009). Therefore, further work on this subject is warranted, namely in what regards the Lf's direct targets on cancer cells.

I.1.2. Clinical Applications of Lactoferrin

Being Lf a multifaceted protein with numerous interesting biological activities, its research advanced from basic research to clinical trials in which encouraging results were achieved. This, together with its established manufacturing process and consequent availability, expanded the Lf clinical potential namely regarding disease prevention, treatment and diagnosis (Tomita et al., 2009).

In the light of several *in vitro* and *in vivo* studies, many human clinical trials have been performed to attest the effectiveness of Lf administration against a large variety of human pathologies (reviewed by Rodrigues et al., 2009). Specifically on cancer therapeutics, some Lf forms have been investigated for the treatment of diverse types of cancer. In 2002, Morinaga Milk Industry Co Ltd. supported a human clinical trial, to determine whether oral intake of bLf would inhibit the growth of adenomatous colorectal polyps in human patients. It was found that a 1-year oral intake of 3 g of bLf per day induced statistically significant retardation of colorectal adenomatous polyp size in participants 63 years-old or younger (Kozu et al., 2009). Also, another company, Agennix, tested a recombinant form of human lactoferrin (talactoferrin), in phase II clinical trials for the treatment of non-small cell lung cancer. It was concluded that talactoferrin in combination with carboplatin and paclitaxel, revealed an apparent improvement in overall patient survival (Digumarti et al., 2011). Other clinical trials tested talolactoferrin in patients with various types of solid tumors concluding that it might be particularly effective against metastatic renal carcinoma and non-small cell lung cancer (Hayes et al., 2006, 2010; Jonasch et al., 2008).

The forms of Lf that have been mostly used in the field of health care and disease are Lf from bovine origin (bLf) and the recombinant talactoferrin referred above. bLf can easily be isolated from cow's milk and it is nowadays produced by diverse manufacturing companies mainly by a cation-exchange chromatography system (Tomita et al., 2009). bLf has already been approved by the European Food Safety Authority as a safe ingredient for various applications, including for medical purposes, since no adverse effects were reported in several studies with humans (EFSA-NDA, 2012). Talactoferrin is produced in *Aspergillus niger*, a filamentous fungus, and it is structurally identical to the native hLf in all aspects differing only in its glycosylation (Jonasch et al., 2008).

As for the administration, it has been proved that the oral dosage of Lf is the most promising option as the dosing can be conducted easily and safely. Additionally, *in vitro* and *in vivo* digestion studies have demonstrated the Lf gastric survival (Liao et al., 2012). In fact, Troost and co-workers demonstrated that more than 60% of the administrated bLf, after the passage through adult human stomach and entrance in the small intestine, remains intact (Troost et al., 2001). Significant amounts of Lf were also found in fecal samples collected from exclusively breast-fed infants (Davidson and

Lönnerdal, 1987). Currently, Lf delivery systems are being developed in order to improve its clinical utility and also to reduce the dosing amount and the frequency (Onishi, 2011).

On the other hand, given that Lf levels are altered in certain pathologies, it has been considered a reliable biomarker for disease. In fact, the increased levels of fecal Lf were found to be specific for the detection of inflammation in patients with inflammatory bowel disease (Kane et al., 2003) and also to distinguish the aforementioned disease from irritable bowel disease (Zhou et al., 2014). In addition, Lf was suggested to be a potential biomarker for nasopharyngeal carcinoma since it was shown to be downregulated in these tumor cells and its increased expression has been associated with a good prognosis (Zhang et al., 2014c).

Further research on the clinical application of Lf is required, particularly on cancer therapeutics, in order to establish the usefulness of Lf as a pharmaceutical drug to be routinely applied. Therefore, it is very important that the commercial development of Lf goes hand-in-hand with basic research, particularly aiming at the elucidation of specific mechanisms of action (Brock, 2012).

I.2. TUMOR-MICROENVIRONMENT AND ITS ACIDITY

According to the current knowledge, carcinogenesis and tumor progression should be considered not as a cancer cell-centered condition, but rather as a disease involving complex multicellular interactions within a newly formed tissue, the cancer tissue (Lorusso and Rüegg, 2008). It was in this context that the term tumor microenvironment (TME) arose. This concept implies that cancer cells do not manifest the disease alone, but rather interact with normal cell types either physically or by the secretion of signalling molecules. van Kempen et al., 2003 postulated that the TME is the functional and structural collection of neoplastic and non-neoplastic cells, in addition to the dynamic microenvironment in which they live, with the emphasis on their functional interactions.

TME also contributes to the acquisition of cancer hallmark traits and its importance in the regulation of carcinogenesis is well-documented, like for example in the case of breast cancer progression (Kenny et al., 2010; Artacho-cordón et al., 2012; Hanahan and Coussens, 2012). Therefore, it acts as a “soil” in the formation, growth,

survival and metastasis of tumors (Wu et al., 2013). Notably, it seems that this microenvironment emerges during the course of tumorigenesis (Hanahan and Weinberg, 2011) and undergoes extensive changes during the multi-steps of this event (Spano and Zollo, 2012). TME is constituted of non-transformed host stromal cells, known to contribute in important ways to the biology of many tumors, such as endothelial cells, pericytes, cancer-associated fibroblasts, various immune cells, and a complex extracellular matrix (ECM) secreted by both the normal and neoplastic cells embedded in it (Hanahan and Weinberg, 2011; Ding et al., 2012).

The microenvironment of tumors comprises several features such as acidic pH, low nutrient levels, elevated interstitial fluid pressure (IFP), overexpressed proteases, and low levels of oxygenation (hypoxia) that is related to the abnormal vascular network that exists in tumors (Lunt et al., 2009; Wu et al., 2013). This hostile microenvironment provides the necessary signals that turn on several transcription factors resulting in the up-regulation of a great number of gene products known to promote malignant progression and metastatic dissemination (Rofstad et al., 2006). Moreover, there is a high level of heterogeneity in the pathophysiological TME both between different tumors and within an individual tumor (Lunt et al., 2009).

The feature of the TME that will be explored in the scope of this work is the acidic pH. Nowadays, it is known that cancer cells, regardless of their origin and genetic background, have an aberrant regulation of hydrogen ion dynamics leading to a reversal of the intracellular to extracellular pH gradient in tumors. Indeed, non-invasive measurements have shown that the extracellular pH (pHe) in tumors ranges from 6.5 to 6.9, whilst the intracellular pH (pHi) remains neutral to alkaline, creating an acid-outside pH gradient typically not observed in normal tissues (Wojtkowiak et al., 2011). This perturbation in pH dynamics rises very early in carcinogenesis and is one of the most common hallmarks of cancer (Reshkin et al., 2013). Consequently, the slightly acidic pH in the TME has become an important issue in the design of anti-tumor therapies, also because it contributes to the resistance to conventional therapies (Wu et al., 2013)

Tumor acidity is a complex and multifactorial process (Lunt et al., 2009). Although TME acidification can be related to an hypoxia-induced enhanced glycolytic activity that leads to production and secretion of H^+ to the extracellular space (Wojtkowiak et al., 2011), it can also occur independently of hypoxia. Therefore,

acidification may be an intrinsic property of altered tumor cell metabolism, which likely evolved to provide tumor cells with a competitive advantage over stromal cells (Gillies et al., 2002). It appears that this pH dysregulation within TME remodels various physiological functions making solid tumors to become invasive and metastatic, and also contributing to tumor resistance to cytotoxic drugs (Barar and Omidi, 2013). In fact, there are several reports attesting that TME acidity promotes invasive growth and metastatic dissemination. In human melanoma cells it was shown that growth at acidic pHe *in vitro* enhances the potential to form experimental lung metastases in mice (Rofstad et al., 2006). This was also evidenced in the murine cell lines KHT-C2-LP1 fibrosarcoma and B16F1 melanoma where, following exposure to acidosis, cells showed an increase in experimental metastatic ability (Jang and Hill, 1997). Consequently, the concept of acid-mediated metastasis arose (Gatenby et al., 2006) as there are multiples steps of metastasis that seem to exhibit pH-sensitivity (Hashim et al., 2011). Importantly, methods to evaluate the metastatic potential based on intra-tumoral acidosis have been developed, reinforcing the importance of H^+ dynamic in the metastatic process (Wang et al., 2014).

The establishment and maintenance of the acidic TME is a direct consequence of the cancer cells ability to secrete protons (Zhang et al., 2010). This H^+ secretion is driven by a series of proton pumps that are up-regulated in cancer cells also protecting them from intracellular acidity and apoptosis (Bellone et al., 2013). These proton-exporting systems include vacuolar H^+-ATPase, H^+/Na^+ exchangers, carbonic anhydrases (CAs), H^+/Cl^- symporter, monocarboxylate transporters (MCT, mainly MCT1) and Na^+-dependent Cl^-/HCO_3^- exchangers (Zhang et al., 2010; Reshkin et al., 2013). In the present work we are interested in the specific role of V-ATPase in the acidic TME of cancer cells.

I.2.1. V-ATPase and Cancer

Vacuolar H^+-ATPases (V-ATPases) are a family of proton pumps that couple the energy of ATP hydrolysis to actively transport protons across both intracellular and plasma membranes of eukaryotic cells (Cipriano et al., 2008). These pumps are highly phylogenetically conserved among prokaryotes and eukaryotes and its importance is now well-recognized (Lee et al., 2010). Indeed, V-ATPases are crucial for numerous biological functions as they trigger a H^+ transmembrane electrochemical potential that

is used to drive a variety of secondary active transport systems via H^+-dependent symporters and antiporters, and channel-mediated transport systems (Beyenbach and Wieczorek, 2006). Thus, a diverse collection of physiological processes depend on V-ATPases, and a number of diseases have been associated with anomalies of these pumps (Bowman and Bowman, 2005).

As for its structure, V-ATPase is a large multi-subunit complex organized into two major functional domains known as V_1 and V_o. The V_1 domain is soluble and comprises at least eight different subunits (A–H). This domain contains three catalytic sites for ATP hydrolysis formed by A and B subunits. The V_o is a membrane-bound domain that contains up to five subunits (*a*, *c*, *c′*, *c′′* and *d*) and is responsible for proton translocation across the membranes (Fig. I.4) (Sun-Wada et al., 2004; Saroussi and Nelson, 2009). V-ATPase operates by a rotary mechanism that is driven by the hydrolysis of ATP within the V_1 domain (Cipriano et al., 2008).

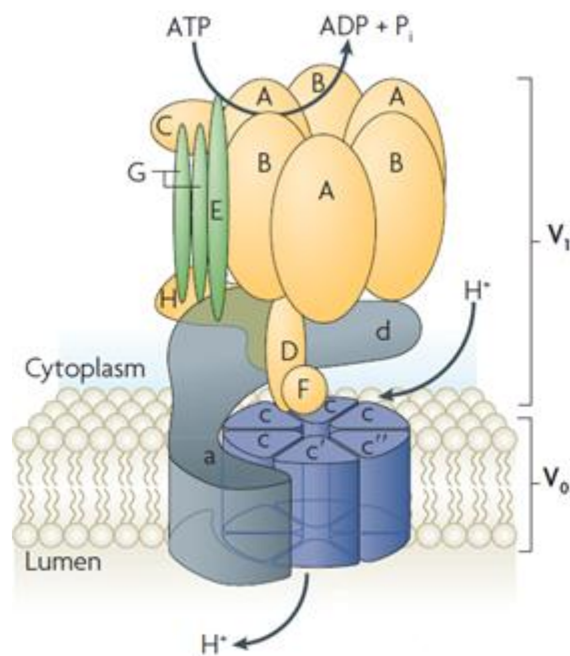


Figure I.4: Structure of V-ATPase (adapted from Casey et al., 2010).

V-ATPase was firstly discovered in lysosomes and in the central vacuoles of fungi and plants. Afterwards, it was identified in secretory vesicles, clathrin-coated vesicles, endosomes, Golgi-derived vesicles, among many others (Saroussi and Nelson, 2009). Besides its intracellular localization, V-ATPase is also present in plasma membrane of

cancer cells, as well as in a variety of specialized cells like osteoclasts, epididymal clear cells, renal alpha-intercalated cells, neutrophils and macrophages (Jefferies et al., 2008).

Focusing on cancer cell V-ATPase, it has been reported that this pump is overexpressed and localized at the plasma membrane in many types of metastatic cancers such as sarcomas (Perut et al., 2013), breast carcinomas (Sennoune et al., 2004) and melanomas (Baruthio et al., 2008). Moreover, highly metastatic cells were shown to preferentially use plasma membrane V-ATPase over Na^+/H^+ exchangers (Salyer et al., 2013). Authors have suggested that such an abnormal localization is much likely one of the initial steps of malignant cells transformation and its aberrant functioning, a continual enhancer of carcinogenesis and tumor progression (Lu and Qin, 2012). In this basis, the targeting of V-ATPase to the cell surface has been proposed to both contribute to the alkalinization of the tumor cell cytoplasm and to the acidification of the extracellular TME that aids invasion (Hinton et al., 2009; Pérez-Sayáns et al., 2009). The most important roles of V-ATPase in tumors are in cellular invasiveness, angiogenesis, proliferation, tumorigenesis and drug resistance (Boyd et al., 2001; Spugnini et al., 2010). However, it is in the metastatic process that V-ATPase has demonstrated major importance. Sennoune et al., 2004 demonstrated the plasma membrane localization of V-ATPase and its greater activity in highly than in lowly metastatic human breast cancer cells. The authors concluded that V-ATPase preferential expression at the cell surface is important for the acquisition of invasive and metastatic potential. *a3* and *a4* V-ATPase subunits isoforms were shown to be responsible for the translocation of this pump to the cell surface (Hinton et al., 2009; Capecci and Forgac, 2013). One fact that can explain the relevance of V-ATPase for tumor metastasis is that, by maintaining the aberrant acidic pH in the TME, it contributes to the activation, secretion, and cellular distribution of many proteases involved in the digestion of ECM including matrix metalloproteinases (MMP) (Fais et al., 2007; Pérez-Sayáns et al., 2009). In fact, in mouse malignant melanoma B16-F10 cells, it was found that the *a3* subunit of V-ATPase promotes distant metastasis by stimulating invasiveness through increasing the expression and activity of MMP-2 and MMP-9 (Nishisho et al., 2011). V-ATPases may also play a role in tumor cell survival through pH regulation, prevention of acidosis-induced apoptosis and promotion of drug resistance (Casey et al., 2010).

More recently, a specific V-ATPase localization at the plasma membrane was proposed. In some cell types, plasma membrane V-ATPase was found to particularly localize at cholesterol-rich lipid rafts. Lipid rafts are microdomains of the cellular membranes that encompass high concentrations of lipids, especially cholesterol and sphingolipids, and also transmembrane or glycosylphosphatidylinisotol-anchored proteins. These structures represent authentic signalling platforms that are essential for signal transduction and protein trafficking, and are resistant to extraction with non-ionic detergents being generally isolated as detergent-resistant membranes (DRMs) (Staubach and Hanisch, 2011). Several lines of evidence indicate that deregulation of raft-dependent signalling favors tumor progression (reviewed by Murai, 2012). V-ATPase was already found to be a component of lipid rafts in highly metastatic melanoma cells (Baruthio et al., 2008), osteoclasts (Ryu et al., 2010), coronary arterial endothelial cells (Xu et al., 2012), synaptic plasma membrane (Yoshinaka et al., 2004), Jurkat T cells (Haller et al., 2001) and monocytic THP-1 cells (Li et al., 2003). In melanoma cells, it was demonstrated that V-ATPase is present in the rafts fraction of highly metastatic cells being otherwise inconspicuous in this fraction in non-metastatic cell lines. The authors suggested that the increased association of V-ATPase with rafts of the metastatic cells may reflect their increased activity (Baruthio et al., 2008). The reason for such a V-ATPase localization is still unknown, however, in coronary arterial endothelial cells, it was shown that V-ATPase is translocated and assembled into the lipid rafts where it provides an acidic microenvironment around these structures that promotes the formation of larger ceramide-enriched signalling platforms and amplifies raft-associated signals. When V-ATPase was inhibited by BafA1 or siRNA, the V-ATPase-mediated acidification was impaired thereby impeding lipid rafts clustering (Xu et al., 2012). In conclusion, when V-ATPase is localized at lipid rafts it is essential for their structural stability and functioning.

Given the V-ATPase importance for cancer cell biology and its substantial impact on pHi and pHe, it constitutes an especially attractive target for anticancer drugs like proton pump inhibitors (PPIs).

I.2.2. Proton Pump Inhibitors (PPIs): a promising anti-cancer therapy

Proton pump inhibitors (PPIs) are lipophilic and weak base pro-drugs that block/downregulate H^+ transporters. They penetrate cell membranes and concentrate in acidic compartments, where they are very unstable being then converted into sulfonamide forms, which represent the active inhibitors (Bellone et al., 2013). Firstly, they were designed to act as potent inhibitors of the gastric acid pump H^+/K^+ ATPase, but later its application was extended to V-ATPase. PPIs have been used for decades as treatment for peptic diseases such as duodenal or gastric ulcers, with minimal side effects (Luciani et al., 2004; De Milito et al., 2010), and there are already various PPIs commercially available worldwide such as omeprazole, lansoprazole, esomeprazole and pantoprazole (De Milito and Fais, 2005).

As previously mentioned, the low extracellular pH of the TME is a hallmark of cancer that is mainly dependent on the activity of proton pumps that are overexpressed in tumor cells. In view of these evidence, these pumps have attracted attention as candidates for anti-cancer therapeutic agents because, by inhibiting them, it is possible to reverse tumor specific H^+ homeostasis, therefore acting on one of "cancer's Achilles' Heel" (Kroemer and Pouyssegur, 2008; Vishvakarma and Singh, 2011). Following this idea, after protonation, PPIs irreversibly bind the proton pump, dramatically disrupting H^+ translocation and dynamics, and limiting TME acidosis (Fais et al., 2007). Thus, in the presence of PPIs, tumor cells are no longer able to control pH and undergo apoptosis which, consequently, results in inhibition of tumor proliferation and growth. The risk of minor adverse effects from PPIs is low, approximately 1-3%, with no significant differences noted between the PPIs (Thomson et al., 2010). PPIs are then perfect suited for a cancer-specific targeted strategy as their activation requires acidity such as that found in the TME (Bellone et al., 2013).

I.2.2.1. Targeting V-ATPase with PPIs

Given the V-ATPase significance and multifunctions in cancer, namely the maintenance of the acidic TME, many specific inhibitors have been developed (Bowman and Bowman, 2005). Actually, there are different classes of V-ATPase inhibitors that can be applied in cancer treatment (Pérez-Sayáns et al., 2009) and there are a plethora of

effects in the cell that have been demonstrated as a consequence of their application, including the variation in cytosolic pH homeostasis, cell cycle arrest, reactive oxygen species (ROS) production, modifications in cell signalling and increased chemosensitivity. These effects, if severe and sustained enough, can lead to cell death mostly apoptotic (Hernández et al., 2012).

Although classic V-ATPase inhibitors (e.g., bafilomycins and concanamycins) or its molecular silencing can induce cell death in tumor cell lines, these compounds are highly toxic and not suitable for clinical use in humans (De Milito et al., 2007). Thus, PPIs have largely and successfully been applied due to their reduced cytotoxicity. The inhibition of V-ATPase with a PPI in cancer cells can have several consequences since the mechanisms that contribute to the malignant behavior are modified. These include neutralization of the TME and consequent apoptosis, reduction of the metastatic potential, chemosensitization and inhibition of tumor growth and survival (Fig. I.5) (Fais et al., 2007; Pérez-Sayáns et al., 2009).

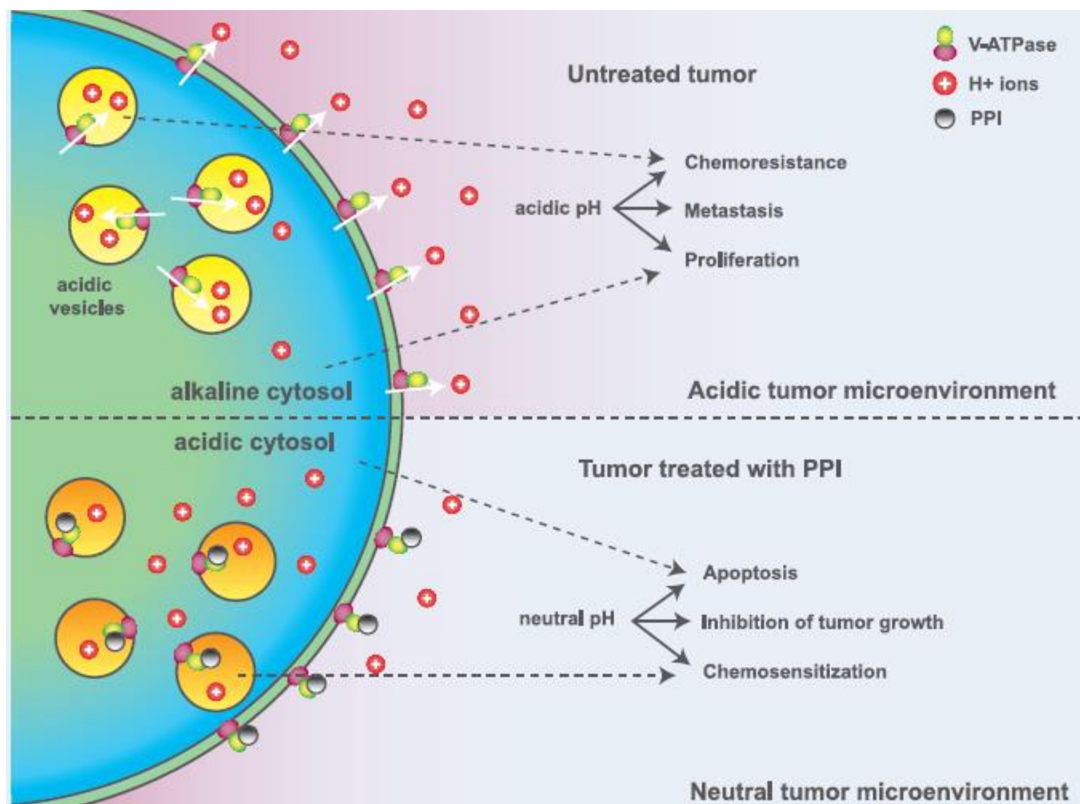


Figure I.5: Effects of V-ATPase inhibition with PPIs on tumor microenvironment (adapted from Fais et al., 2007).

There are some PPIs targeting V-ATPase that have already been tested. De Milito et al., 2010 performed a pre-clinical study in human melanoma cells both *in vitro* and *in vivo*, in which they demonstrated that esomeoprazol, a PPI that inhibits V-ATPase, is capable of inhibiting tumor growth and significantly increase animal survival. On the other hand, pre-treatment with a PPI, through the inhibition of V-ATPase activity, was found to induce susceptibility of various human drug-resistant tumor cell lines to the cytotoxic effect of different anti-tumor drugs, with a marked reduction of drug efflux and decreased tumor acidity. Thus, these drugs can also be used to overcome and/or reverse the multi-resistance of the human tumors (Luciani et al., 2004).

I.3. AIMS

Although several studies have addressed the anti-tumoral activity of Lf, the underlying molecular mechanism is still elusive. Therefore, the general goal of the present study was to unveil the molecular target(s) of Lf in cancer cells. In particular, to explore the potential role of Lf as a PPI, three breast cell lines with distinct features were used, namely the highly metastatic cell line Hs 578T, the non-invasive cell line T-47D, and the non-tumorigenic cell line MCF-10-2A. In order to accomplish our goal, specific aims were design, to:

- investigate the susceptibilities of these three cell lines to bLf regarding cell proliferation and apoptosis, so that we could ascertain whether the susceptibilities were in line with our hypothesis. Also infer about the bLf specificity to cancer cells;
- study the distribution of V-ATPase in the breast cancer cell lines to evaluate if existing differences between the three cell lines can correlate with the observed susceptibilities to bLf;
- assess key cellular events caused by bLf, such as extracellular and intracellular pH, that could be in agreement with the hypothetical V-ATPase inhibition;
- determine the effect of bLf on cholesterol-enriched lipid rafts that were found to contain V-ATPase in some types of metastatic tumor cells;
- provide *in vitro* evidences of the putative V-ATPase inhibition by bLf through the study of both proton pumping or hydrolytic activities.

The outcome of the present work may have great implications on cancer therapy, especially of highly metastatic tumors that are characterized by its acidic microenvironment.

I.4. REFERENCES

Adlerova, L., A. Bartoskova, and M. Faldyna. 2008. Lactoferrin : a review. *Veterinarni Medicina*. 53(9):457–468.

Akiyama, Y., K. Oshima, K. Shin, H. Wakabayashi, F. Abe, D. Nadano, and T. Matsuda. 2013. Intracellular retention and subsequent release of bovine milk lactoferrin taken up by human enterocyte-like cell lines, Caco-2, C2BBe1 and HT-29. *Biosci. Biotechnol. Biochem.* 77:1023–9. doi:10.1271/bbb.121011.

Alexander, D.B., M. Iigo, K. Yamauchi, M. Suzui, and H. Tsuda. 2012. Lactoferrin: an alternative view of its role in human biological fluids. *Biochem. Cell Biol.* 90:279–306. doi:10.1139/o2012-013.

Andrés, M.T., and J.F. Fierro. 2010. Antimicrobial mechanism of action of transferrins: selective inhibition of H^+-ATPase. *Antimicrob. Agents Chemother.* 54:4335–42. doi:10.1128/AAC.01620-09.

Andrés, M.T., M. Viejo-Díaz, and J.F. Fierro. 2008. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K^+-channel-mediated K^+ efflux. *Antimicrob. Agents Chemother.* 52:4081–8. doi:10.1128/AAC.01597-07.

Artacho-cordón, A., F. Artacho-cordón, S. Ríos-arrabal, I. Calvente, and M.I. Núñez. 2012. Tumor microenvironment and breast cancer progression: a complex scenario. *Cancer Biol. Ther.* 13:14–24.

Baker, H.M., and E.N. Baker. 2004. Lactoferrin and Iron: structural and dynamic aspects of binding and release. *BioMetals*. 17:209–216. doi:10.1023/B:BIOM.0000027694.40260.70.

Baker, H.M., and E.N. Baker. 2012. A structural perspective on lactoferrin function. *Biochem. Cell Biol.* 90:320–8. doi:10.1139/o11-071.

Barar, J., and Y. Omidi. 2013. Dysregulated pH in Tumor Microenvironment Checkmates Cancer Therapy. *Bioimpacts*. 3:149–62. doi:10.5681/bi.2013.036.

Baruthio, F., M. Quadroni, C. Rüegg, and A. Mariotti. 2008. Proteomic analysis of membrane rafts of melanoma cells identifies protein patterns characteristic of the tumor progression stage. *Proteomics*. 8:4733–47. doi:10.1002/pmic.200800169.

Baumrucker, C.R., F. Schanbacher, Y. Shang, and M.H. Green. 2006. Lactoferrin interaction with retinoid signaling: cell growth and apoptosis in mammary cells. *Domest. Anim. Endocrinol.* 30:289–303. doi:10.1016/j.domaniend.2005.07.009.

Bellone, M., A. Calcinotto, P. Filipazzi, A. De Milito, S. Fais, and L. Rivoltini. 2013. The acidity of the tumor microenvironment is a mechanism of immune escape that can be overcome by proton pump inhibitors. *Oncoimmunology*. 2:e22058. doi:10.4161/onci.22058.

Berkel, P.H.C.V.A.N., M.E.J. Geerts, H.A.V.A.N. Veen, M. Mericskay, H.A.D.E. Boer, and J.H. Nuijens. 1997. N-terminal stretch Arg 2 , Arg 3 , Arg 4 and Arg 5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. *Biochem. J.* 328:145–151.

Berlutti, F., F. Pantanella, T. Natalizi, A. Frioni, R. Paesano, A. Polimeni, and P. Valenti. 2011. Antiviral properties of lactoferrin-a natural immunity molecule. *Molecules*. 16:6992–7018. doi:10.3390/molecules16086992.

Beyenbach, K.W., and H. Wieczorek. 2006. The V-type H^+ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* 209:577–89. doi:10.1242/jeb.02014.

Bezault, J., R. Bhimani, and J. Wiprovnick. 1994. Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice. *Cancer Res.* 54:2310–2312.

Bowman, E.J., and B.J. Bowman. 2005. V-ATPases as drug targets. *J. Bioenerg. Biomembr.* 37:431–5. doi:10.1007/s10863-005-9485-9.

Boyd, M.R., C. Farina, P. Belfiore, S. Gagliardi, J.I.N.W.O.O. Kim, Y. Hayakawa, J.A. Beutler, T.C. Mckee, B.J. Bowman, and E.J. Bowman. 2001. Discovery of a Novel Antitumor Benzolactone Enamide Class That Selectively Inhibits Mammalian Vacuolar-Type (H^+) -ATPases. *J. Pharmacol. Exp. Ther.* 297:114–120.

Brock, J.H. 2012. Lactoferrin-50 years on. *Biochem. Cell Biol.* 90:245–51. doi:10.1139/o2012-018.

Buccigrossi, V., G. de Marco, E. Bruzzese, L. Ombrato, I. Bracale, G. Polito, and A. Guarino. 2007. Lactoferrin induces concentration-dependent functional modulation of intestinal proliferation and differentiation. *Pediatr. Res.* 61:410–4. doi:10.1203/pdr.0b013e3180332c8d.

Capecci, J., and M. Forgac. 2013. The function of vacuolar ATPase (V-ATPase) *a* subunit isoforms in invasiveness of MCF10a and MCF10CA1a human breast cancer cells. *J. Biol. Chem.* 288:32731–41. doi:10.1074/jbc.M113.503771.

Casey, J.R., S. Grinstein, and J. Orlowski. 2010. Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell Biol.* 11:50–61. doi:10.1038/nrm2820.

Cipriano, D.J., Y. Wang, S. Bond, A. Hinton, K.C. Jefferies, J. Qi, and M. Forgac. 2008. Structure and regulation of the vacuolar ATPases. *Biochim. Biophys. Acta*. 1777:599–604. doi:10.1016/j.bbabio.2008.03.013.

Cumberbatch, M., M. Bhushan, R.J. Dearman, I. Kimber, C.E.M.G. Syngenta, and C. Toxicology. 2003. IL-1β -induced Langerhans ' cell migration and TNF-α production in human skin : regulation by lactoferrin. *Clin Exp Immunol*. 132:352–359.

Damiens, E., I. El Yazidi, J. Mazurier, I. Duthille, G. Spik, and Y. Boilly-Marer. 1999. Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. *J. Cell. Biochem.* 74:486–98.

Dashper, S.G., Y. Pan, P.D. Veith, Y.-Y. Chen, E.C.Y. Toh, S.W. Liu, K.J. Cross, and E.C. Reynolds. 2012. Lactoferrin inhibits *Porphyromonas gingivalis* proteinases and has sustained biofilm inhibitory activity. *Antimicrob. Agents Chemother.* 56:1548–56. doi:10.1128/AAC.05100-11.

Davidson, L.A., and B. Lönnerdal. 1987. Persistence of Human Milk Proteins in the Breast-Fed Infant. *Acta Paediatr.* 76:733–740. doi:10.1111/j.1651-2227.1987.tb10557.x.

Day, C.L., K.M. Stowell, E.N. Baker, and W. Tweediell. 1992. Studies of the N-terminal Half of Human Lactoferrin Produced from the Cloned cDNA Demonstrate That Interlobe Interactions Modulate Iron Release. *J. Biol. Chem.* 267:13857–13862.

Deng, M., W. Zhang, H. Tang, Q. Ye, Q. Liao, Y. Zhou, M. Wu, W. Xiong, Y. Zheng, X. Guo, Z. Qin, W. He, M. Zhou, J. Xiang, X. Li, J. Ma, and G. Li. 2013. Lactotransferrin acts as a tumor suppressor in nasopharyngeal carcinoma by repressing AKT through multiple mechanisms. *Oncogene*. 32:4273–83. doi:10.1038/onc.2012.434.

Diarra, M.S., D. Petitclerc, É. Deschênes, N. Lessard, G. Grondin, B.G. Talbot, and P. Lacasse. 2003. Lactoferrin against *Staphylococcus aureus* Mastitis. *Vet. Immunol. Immunopathol.* 95:33–42. doi:10.1016/S0165-2427(03)00098-9.

Digumarti, R., Y. Wang, G. Raman, D.C. Doval, S.H. Advani, P.K. Julka, P.M. Parikh, S. Patil, S. Nag, J. Madhavan, A. Bapna, A.A. Ranade, A. Varadhachary, and R. Malik. 2011. A randomized, double-blind, placebo-controlled, phase II study of oral talactoferrin in combination with carboplatin and paclitaxel in previously untreated locally advanced or metastatic non-small cell lung cancer. *J Thorac Oncol*. 6:1098–103.

Ding, Z.-Y., X.-L. Zou, and Y.-Q. Wei. 2012. Cancer microenvironment and cancer vaccine. *Cancer Microenviron.* 5:333–44. doi:10.1007/s12307-012-0107-x.

Duarte, D.C., a Nicolau, J. a Teixeira, and L.R. Rodrigues. 2011. The effect of bovine milk lactoferrin on human breast cancer cell lines. *J. Dairy Sci.* 94:66–76. doi:10.3168/jds.2010-3629.

EFSA-NDA, European Food Safety Authority - Dietetic Products, Nutrition and Allergies 2012. Scientific opinon on bovine lactoferrin. *EFSA J.* 10.

El-Fakharany, E.M., L. Sánchez, H. a Al-Mehdar, and E.M. Redwan. 2013. Effectiveness of human, camel, bovine and sheep lactoferrin on the hepatitis C virus cellular infectivity: comparison study. *Virol. J.* 10:199. doi:10.1186/1743-422X-10-199.

Fais, S., A. De Milito, H. You, and W. Qin. 2007. Targeting vacuolar H^+-ATPases as a new strategy against cancer. *Cancer Res.* 67:10627–30. doi:10.1158/0008-5472.CAN-07-1805.

Fujita, K., E. Matsuda, K. Sekine, M. Iigo, and H. Tsuda. 2004. Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats. *Carcinogenesis*. 25:1961–6. doi:10.1093/carcin/bgh205.

García-Montoya, I.A., T.S. Cendón, S. Arévalo-Gallegos, and Q. Rascón-Cruz. 2012. Lactoferrin a multiple bioactive protein: an overview. *Biochim. Biophys. Acta*. 1820:226–36. doi:10.1016/j.bbagen.2011.06.018.

Gatenby, R. a, E.T. Gawlinski, A.F. Gmitro, B. Kaylor, and R.J. Gillies. 2006. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res.* 66:5216–23. doi:10.1158/0008-5472.CAN-05-4193.

Gibbons, J.A., R.K. Kanwar, and J.R. Kanwar. 2011. Lactoferrin and cancer in different cancer models. *Front Biosci (Schol Ed)*. 3:1080–8.

Gifford, J.L., H. Ishida, and H.J. Vogel. 2012. Structural characterization of the interaction of human lactoferrin with calmodulin. *PLoS One*. 7:e51026. doi:10.1371/journal.pone.0051026.

Gillies, R.J., N. Raghunand, G.S. Karczmar, and Z.M. Bhujwalla. 2002. MRI of the tumor microenvironment. *J. Magn. Reson. Imaging*. 16:430–50. doi:10.1002/jmri.10181.

González-Chávez, S. a, S. Arévalo-Gallegos, and Q. Rascón-Cruz. 2009. Lactoferrin: structure, function and applications. *Int. J. Antimicrob. Agents*. 33:301.e1–8. doi:10.1016/j.ijantimicag.2008.07.020.

Gorria, M., X. Tekpli, M. Rissel, O. Sergent, L. Huc, N. Landvik, O. Fardel, M.-T. Dimanche-Boitrel, J. a Holme, and D. Lagadic-Gossmann. 2008. A new lactoferrin- and iron-dependent lysosomal death pathway is induced by benzo[a]pyrene in hepatic epithelial cells. *Toxicol. Appl. Pharmacol.* 228:212–24. doi:10.1016/j.taap.2007.12.021.

Groves, M.L. 1960. The isolation of a red protein from milk. *J Am Chem Soc*. 82:3345–3350.

Haller, P.D. Von, S. Donohoe, J.D. Watts, and D.R. Goodlett. 2001. Mass spectrometric characterization of proteins extracted from Jurkat T cell detergent-resistant membrane domains. 193:181–193.

Hanahan, D., and L.M. Coussens. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 21:309–22. doi:10.1016/j.ccr.2012.02.022.

Hanahan, D., and R. a Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646–74. doi:10.1016/j.cell.2011.02.013.

Hashim, A.I., X. Zhang, J.W. Wojtkowiakk, and R.J. Gillies. 2011. Imaging pH and Metastasis. *NMR Biomed*. 24:582–591. doi:10.1002/nbm.1644.Imaging.

Hayes, T.G., G.F. Falchook, G.R. Varadhachary, D.P. Smith, L.D. Davis, H.M. Dhingra, B.P. Hayes, and A. Varadhachary. 2006. Phase I trial of oral talactoferrin alfa in refractory solid tumors. *Invest. New Drugs*. 24:233–40. doi:10.1007/s10637-005-3690-6.

Hayes, T.G., G.S. Falchook, and A. Varadhachary. 2010. Phase IB trial of oral talactoferrin in the treatment of patients with metastatic solid tumors. *Invest. New Drugs*. 28:156–62. doi:10.1007/s10637-009-9233-9.

He, J., and P. Furmanski. 1995. Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. *Nature*. 373:721–724.

Hendrixson, D.R., J. Qiu, S.C. Shewry, D.L. Fink, S. Petty, E.N. Baker, A.G. Plaut, and J.W.S.G. Iii. 2003. Human milk lactoferrin is a serine protease that cleaves Haemophilus surface proteins at arginine-rich sites. *Mol. Microbiol.* 47:607–617.

Hernández, A., G. Serrano-bueno, J.R. Pérez-castiñeira, and A. Serrano. 2012. Intracellular Proton Pumps as Targets in Chemotherapy : V-ATPases and Cancer. *Curr. Pharm. Des.* 18:1383–1394.

Hinton, A., S.R. Sennoune, S. Bond, M. Fang, M. Reuveni, G.G. Sahagian, D. Jay, R. Martinez-Zaguilan, and M. Forgac. 2009. Function of a subunit isoforms of the V-ATPase in pH homeostasis and in vitro invasion of MDA-MB231 human breast cancer cells. *J. Biol. Chem.* 284:16400–8. doi:10.1074/jbc.M901201200.

Hoedt, E., K. Chaoui, I. Huvent, C. Mariller, B. Monsarrat, O. Burlet-schiltz, and A. Pierce. 2014. SILAC-Based Proteomic Profiling of the Human MDA-MB- 231 Metastatic Breast Cancer Cell Line in Response to the Two Antitumoral Lactoferrin Isoforms : The Secreted Lactoferrin and the Intracellular Delta-Lactoferrin. *PLos One*. 9. doi:10.1371/journal.pone.0104563.

Iigo, M., T. Kuhara, Y. Ushida, K. Sekine, and M.A. Moore. 1999. Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. *Clin Exp Metastasis*. 35–40.

Iigo, M., M. Shimamura, E. Matsuda, K. Fujita, H. Nomoto, J. Satoh, S. Kojima, D.B. Alexander, M. a Moore, and H. Tsuda. 2004. Orally administered bovine lactoferrin induces caspase-1 and interleukin-18 in the mouse intestinal mucosa: a possible explanation for inhibition of carcinogenesis and metastasis. *Cytokine*. 25:36–44. doi:10.1016/j.cyto.2003.09.009.

Jang, A., and R.P. Hill. 1997. An examination of the effects of hypoxia , acidosis , and glucose starvation on the expression of metastasis-associated genes in murine tumor cells. *Clin. Exp. Metastasis*. 15:469–483.

Jefferies, K.C., D.J. Cipriano, and M. Forgac. 2008. Function, structure and regulation of the vacuolar (H+)-ATPases. *Arch Biochem Biophys.* 476:33–42. doi:10.1016/j.abb.2008.03.025.Function.

Jenssen, H., and R.E.W. Hancock. 2009. Antimicrobial properties of lactoferrin. *Biochimie*. 91:19–29. doi:10.1016/j.biochi.2008.05.015.

Johansson, B. 1960. Isolation of an iron-containing red protein from human milk. *Acta Chem Scand*. 14:510–512.

Jonasch, E., W.M. Stadler, R.M. Bukowski, T.G. Hayes, A. Varadhachary, R. Malik, R. a Figlin, and S. Srinivas. 2008. Phase 2 trial of talactoferrin in previously treated patients with metastatic renal cell carcinoma. *Cancer*. 113:72–7. doi:10.1002/cncr.23519.

Kane, S., W.J. Sandborn, P.A. Rufo, A. Zholudev, J. Bonne, D. Lyerly, M. Camilleri, and S.B. Hanauer. 2003. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. *Am. J. Gastroenterol.* 98:1309–1314. doi:10.1016/S0002-9270(03)00232-6.

Kanwar, J.R., K.P. Palmano, X. Sun, R.K. Kanwar, R. Gupta, N. Haggarty, A. Rowan, S. Ram, and G.W. Krissansen. 2008. "Iron-saturated" lactoferrin is a potent natural adjuvant for augmenting cancer chemotherapy. *Immunol. Cell Biol.* 86:277–88. doi:10.1038/sj.icb.7100163

Kanyshkova, T.G., S.E. Babina, D. V. Semenov, N. Isaeva, A. V. Vlassov, K.N. Neustroev, A. a. Kul'minskaya, V.N. Buneva, and G. a. Nevinsky. 2003. Multiple enzymic activities of human milk lactoferrin. *Eur. J. Biochem.* 270:3353–3361. doi:10.1046/j.1432-1033.2003.03715.

Katunuma, N., Q.T. Le, E. Murata, A. Matsui, E. Majima, N. Ishimaru, Y. Hayashi, and A. Ohashi. 2006. A novel apoptosis cascade mediated by lysosomal lactoferrin and its participation in hepatocyte apoptosis induced by D-galactosamine. *FEBS Lett.* 580:3699–705. doi:10.1016/j.febslet.2006.05.057.

Katunuma, N., E. Murata, Q.T. Le, Y. Hayashi, and A. Ohashi. 2004. New apoptosis cascade mediated by lysosomal enzyme and its protection by epigallo-catechin gallate. *Adv. Enzyme Regul.* 44:1–10. doi:10.1016/j.advenzreg.2003.11.016.

van Kempen, C.L.T., D.J. Ruiter, G.N.P. van Muijen, and L.M. Coussens. 2003. The tumor microenvironment : a critical determinant of neoplastic evolution. *Eur. J. Cell Biol.* 82:539–548.

Kenny, P.A., G.Y. Lee, and M.J. Bissell. 2010. Targeting the tumor microenvironment. *Front Biosci*. 12:3468–3474.

Kozu, T., G. Iinuma, Y. Ohashi, Y. Saito, T. Akasu, D. Saito, D.B. Alexander, M. Iigo, T. Kakizoe, and H. Tsuda. 2009. Effect of orally administered bovine lactoferrin on the growth of adenomatous colorectal polyps in a randomized, placebo-controlled clinical trial. *Cancer Prev. Res.* 2:975–83. doi:10.1158/1940-6207.CAPR-08-0208.

Kroemer, G., and J. Pouyssegur. 2008. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell*. 13:472–82. doi:10.1016/j.ccr.2008.05.005.

De la Rosa, G., D. Y, P. Tewary, A. Varadhachary, and J.J. Oppenheim. 2008. Lactoferrin acts as an alarmin to promote the recruitment and activation of antigen-presenting cells and antigen-specific immune responses. *J Immunol*. 180:6868–6876.

Lambert, L. a, H. Perri, P.J. Halbrooks, and A.B. Mason. 2005. Evolution of the transferrin family: conservation of residues associated with iron and anion binding. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 142:129–41. doi:10.1016/j.cbpb.2005.07.007.

Lee, S., S. Won, C. Pyo, N. Yoo, J. Kim, and S. Choi. 2009. Requirement of the JNK-associated Bcl-2 pathway for human lactoferrin-induced apoptosis in the Jurkat leukemia T cell line. *Biochimie*. 91:102–108. doi:10.1016/j.biochi.2008.05.004.

Lee, S.-K., W. Li, S.-E. Ryu, T. Rhim, and J. Ahnn. 2010. Vacuolar (H+)-ATPases in *Caenorhabditis elegans*: what can we learn about giant H^+ pumps from tiny worms? *Biochim. Biophys. Acta*. 1797:1687–95. doi:10.1016/j.bbabio.2010.07.004.

Legrand, D., and J. Mazurier. 2010. A critical review of the roles of host lactoferrin in immunity. *Biometals*. 23:365–76. doi:10.1007/s10534-010-9297-1.

Legrand, D., A. Pierce, E. Elass, M. Carpentier, C. Mariller, and J. Mazurier. 2008. Lactoferrin structure and functions. *Adv. Exp. Med. Biol.* 606:163–94. doi:10.1007/978-0-387-74087-4_6.

Levay, P.F., and M. Viljoen. 1995. Lactoferrin: a general review. *Haematologica*. 80:252–267.

Li, N., A. Mak, D.P. Richards, C. Naber, B.O. Keller, L. Li, and A.R.E. Shaw. 2003. Monocyte lipid rafts contain proteins implicated in vesicular trafficking and phagosome formation. *Proteomics*. 3:536–48. doi:10.1002/pmic.200390067.

Liao, Y., R. Jiang, and B. Lönnerdal. 2012. Biochemical and molecular impacts of lactoferrin on small intestinal growth and development during early life. *Biochem. Cell Biol.* 90:476–84. doi:10.1139/o11-075.

Ling, J.M.L., and A.B. Schryvers. 2006. Perspectives on interactions between lactoferrin and bacteria. *Biochem. Cell Biol.* 84:275–81. doi:10.1139/o06-044.

Lopez, V., S.L. Kelleher, and B. Lonnerdal. 2008. Lactoferrin receptor mediates apo- but not holo-lactoferrin internalization via clathrin-mediated endocytosis in trophoblasts. *Biochem. J.* 278:271–278. doi:10.1042/BJ20070393.

Lorusso, G., and C. Rüegg. 2008. The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochem. Cell Biol.* 130:1091–103. doi:10.1007/s00418-008-0530-8.

Lu, X., and W. Qin. 2012. Vacuolar H(+)-ATPase in Cancer Cells: Structure and Function. *Atlas Genet. Cytogenet. Oncol. Haematol.* 16:252–259. doi:10.4267/2042/47290.

Luciani, F., M. Spada, A. De Milito, A. Molinari, L. Rivoltini, A. Montinaro, M. Marra, L. Lugini, M. Logozzi, F. Lozupone, C. Federici, E. Iessi, G. Parmiani, G. Arancia, F. Belardelli, and S. Fais. 2004. Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. *J. Natl. Cancer Inst.* 96:1702–13. doi:10.1093/jnci/djh305.

Lunt, S.J., N. Chaudary, and R.P. Hill. 2009. The tumor microenvironment and metastatic disease. *Clin. Exp. Metastasis*. 26:19–34. doi:10.1007/s10585-008-9182-2.

Masson, P.L., J.F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J Exp Med*. 130:643–58.

Masuda, C., H. Wanibuchi, K. Sekine, Y. Yano, S. Otani, T. Kishimoto, H. Tsuda, and S. Fukushima. 2000. Chemopreventive Effects of Bovine Lactoferrin on *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine-induced Rat Bladder Carcinogenesis. *Jpn. J. Cancer Res.* 91:582–588.

Mclntosh, G.H., G.O. Regester, P.J. Royle, and G.W. Smithers. 1995. Biochemical and Molecular Roles of Nutrients Dairy Proteins Protect against Dimethylhydrazine-Induced Intestinal Cancers in Rats1-2. *J. Nutr.* 809–816.

Meier, P., and K.H. Vousden. 2007. Lucifer's labyrinth--ten years of path finding in cell death. *Mol. Cell*. 28:746–54. doi:10.1016/j.molcel.2007.11.016.

De Milito, A., R. Canese, M.L. Marino, M. Borghi, M. Iero, A. Villa, G. Venturi, F. Lozupone, E. Iessi, M. Logozzi, P. Della Mina, M. Santinami, M. Rodolfo, F. Podo, L. Rivoltini, and S. Fais. 2010. pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor acidity. *Int. J. Cancer*. 127:207–19. doi:10.1002/ijc.25009.

De Milito, A., and S. Fais. 2005. Tumor acidity, chemoresistance and proton pump inhibitors. *Future Oncol.* 1:779–86. doi:10.2217/14796694.1.6.779.

De Milito, A., E. Iessi, M. Logozzi, F. Lozupone, M. Spada, M.L. Marino, C. Federici, M. Perdicchio, P. Matarrese, L. Lugini, A. Nilsson, and S. Fais. 2007. Proton pump inhibitors induce apoptosis of human B-cell tumors through a caspase-independent

mechanism involving reactive oxygen species. *Cancer Res.* 67:5408–17. doi:10.1158/0008-5472.CAN-06-4095.

Mizutani, K., M. Toyoda, and B. Mikami. 2012. X-ray structures of transferrins and related proteins. *Biochim. Biophys. Acta*. 1820:203–11. doi:10.1016/j.bbagen.2011.08.003.

Mohan, K., R. Kumaraguruparan, D. Prathiba, and S. Nagini. 2006. Modulation of xenobiotic-metabolizing enzymes and redox status during chemoprevention of hamster buccal carcinogenesis by bovine lactoferrin. *Nutrition*. 22:940–6. doi:10.1016/j.nut.2006.05.017.

Murai, T. 2012. The role of lipid rafts in cancer cell adhesion and migration. *Int. J. Cell Biol.* 2012:763283. doi:10.1155/2012/763283.

Nishisho, T., K. Hata, M. Nakanishi, Y. Morita, G.-H. Sun-Wada, Y. Wada, N. Yasui, and T. Yoneda. 2011. The *a3* isoform vacuolar type H^+-ATPase promotes distant metastasis in the mouse B16 melanoma cells. *Mol. Cancer Res.* 9:845–55. doi:10.1158/1541-7786.MCR-10-0449.

Norrby, K. 2004. Human apo-lactoferrin enhances angiogenesis mediated by vascular endothelial growth factor A *in vivo*. *J. Vasc. Res.* 41:293–304. doi:10.1159/000078927.

Norrby, K., I. Matisby-Baltzer, M. Innocenti, and S. Tuneberg. 2001. Orally administered bovine lactoferrin systematically inhibits VEGF-165-mediated angiogenesis in the rat. *Int. J. Cancer*. 91:236–240.

Oh, S.-M., C.-W. Pyo, Y. Kim, and S.-Y. Choi. 2004. Neutrophil lactoferrin upregulates the human p53 gene through induction of NF-kappaB activation cascade. *Oncogene*. 23:8282–91. doi:10.1038/sj.onc.1208021.

Onishi, H. 2011. Lactoferrin delivery systems: approaches for its more effective use. *Expert Opin. Drug Deliv.* 8:1469–79. doi:10.1517/17425247.2011.615829.

Pérez-Sayáns, M., J.M. Somoza-Martín, F. Barros-Angueira, J.M.G. Rey, and A. García-García. 2009. V-ATPase inhibitors and implication in cancer treatment. *Cancer Treat. Rev.* 35:707–13. doi:10.1016/j.ctrv.2009.08.003.

Perut, F., S. Avnet, C. Fotia, S.R. Baglìo, M. Salerno, S. Hosogi, K. Kusuzaki, and N. Baldini. 2013. V-ATPase as an effective therapeutic target for sarcomas. *Exp. Cell Res.* 320:21–32. doi:10.1016/j.yexcr.2013.10.011.

Puddu, P., P. Valenti, and S. Gessani. 2009. Immunomodulatory effects of lactoferrin on antigen presenting cells. *Biochimie*. 91:11–8. doi:10.1016/j.biochi.2008.05.005.

Reshkin, S.J., R.A. Cardone, and S. Harguindey. 2013. Na^+-H^+ Exchanger, pH Regulation and Cancer. *Recent Pat. Anticancer. Drug Discov.* 8:85–99.

Rodrigues, L., J. Teixeira, F. Schmitt, M. Paulsson, and H.L. Månsson. 2009. Lactoferrin and cancer disease prevention. *Crit. Rev. Food Sci. Nutr.* 49:203–17. doi:10.1080/10408390701856157.

Rofstad, E.K., B. Mathiesen, K. Kindem, and K. Galappathi. 2006. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res.* 66:6699–707. doi:10.1158/0008-5472.CAN-06-0983.

Roseanu, A., P.E. Florian, M. Moisei, L.E. Sima, R.W. Evans, and M. Trif. 2010. Liposomalization of lactoferrin enhanced its anti-tumoral effects on melanoma cells. *Biometals*. 23:485–92. doi:10.1007/s10534-010-9312-6.

Ryu, J., H. Kim, E.-J. Chang, H.J. Kim, Y. Lee, and H.-H. Kim. 2010. Proteomic analysis of osteoclast lipid rafts: the role of the integrity of lipid rafts on V-ATPase activity in osteoclasts. *J. Bone Miner. Metab.* 28:410–7. doi:10.1007/s00774-009-0150-y.

Salyer, S. a, J.R. Olberding, A. a Distler, E.D. Lederer, B.J. Clark, N. a Delamere, and S.J. Khundmiri. 2013. Vacuolar ATPase driven potassium transport in highly metastatic breast cancer cells. *Biochim. Biophys. Acta*. 1832:1734–43. doi:10.1016/j.bbadis.2013.04.023.

Saroussi, S., and N. Nelson. 2009. The little we know on the structure and machinery of V-ATPase. *J. Exp. Biol.* 212:1604–10. doi:10.1242/jeb.025866.

Sennoune, S.R., K. Bakunts, G.M. Martínez, J.L. Chua-Tuan, Y. Kebir, M.N. Attaya, and R. Martínez-Zaguilán. 2004. Vacuolar H^+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. *Am. J. Physiol. Cell Physiol.* 286:C1443–52. doi:10.1152/ajpcell.00407.2003.

Shi, H., and W. Li. 2014. Inhibitory effects of human lactoferrin on U14 cervical carcinoma through upregulation of the immune response. *Oncol. Lett.* 7:820–826. doi:10.3892/ol.2013.1776.

Shimamura, M., Y. Yamamoto, H. Ashino, T. Oikawa, T. Hazato, H. Tsuda, and M. Iigo. 2004. Bovine lactoferrin inhibits tumor-induced angiogenesis. *Int. J. Cancer*. 111:111–6. doi:10.1002/ijc.20187.

Soerensen, M. and Soerensen, S.P.L. 1939. The proteins in whey. *C R Trav Lab Carlsb.* 23:55–99.

Son, H.-J., S.-H. Lee, and S.-Y. Choi. 2006. Human lactoferrin controls the level of retinoblastoma protein and its activity. *Biochem. Cell Biol.* 84:345–50. doi:10.1139/o06-048.

Spano, D., and M. Zollo. 2012. Tumor microenvironment: a main actor in the metastasis process. *Clin. Exp. Metastasis*. 29:381–95. doi:10.1007/s10585-012-9457-5.

Spugnini, E.P., G. Citro, and S. Fais. 2010. Proton pump inhibitors as anti vacuolar-ATPases drugs: a novel anticancer strategy. *J. Exp. Clin. Cancer Res.* 29:44. doi:10.1186/1756-9966-29-44.

Staubach, S., and F.-G. Hanisch. 2011. Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert Rev. Proteomics*. 8:263–77. doi:10.1586/epr.11.2.

Sun, X., R. Jiang, A. Przepiorski, S. Reddy, K.P. Palmano, and G.W. Krissansen. 2012. “Iron-saturated” bovine lactoferrin improves the chemotherapeutic effects of tamoxifen in the treatment of basal-like breast cancer in mice. *BMC Cancer*. 12:591. doi:10.1186/1471-2407-12-591.

Sun-Wada, G.-H., Y. Wada, and M. Futai. 2004. Diverse and essential roles of mammalian vacuolar-type proton pump ATPase: toward the physiological understanding of inside acidic compartments. *Biochim. Biophys. Acta*. 1658:106–14. doi:10.1016/j.bbabio.2004.04.013.

Suzuki, Y. a, V. Lopez, and B. Lönnerdal. 2005. Mammalian lactoferrin receptors: structure and function. *Cell. Mol. Life Sci.* 62:2560–75. doi:10.1007/s00018-005-5371-1.

Thomson, A.B., M.D. Sauve, N. Kassam, and H. Kamitakahara. 2010. Safety of the long-term use of proton pump inhibitors. *World J. Gastroenterol.* 16:2323. doi:10.3748/wjg.v16.i19.2323.

Tomita, M., H. Wakabayashi, K. Shin, K. Yamauchi, T. Yaeshima, and K. Iwatsuki. 2009. Twenty-five years of research on bovine lactoferrin applications. *Biochimie*. 91:52–7. doi:10.1016/j.biochi.2008.05.021.

Toyokuni, S. 2009. Role of iron in carcinogenesis: cancer as a ferrotoxic disease. *Cancer Sci.* 100:9–16. doi:10.1111/j.1349-7006.2008.01001.x.

Troost, F., J. Steijns, W. Saris, Brummer, and M. Robert-jan. 2001. Gastric Digestion of Bovine Lactoferrin In Vivo in Adults. *J. Nutr.* 1:2101–2104.

Tsuda, H., T. Kozu, G. Iinuma, and Y. Ohashi. 2010. Cancer prevention by bovine lactoferrin : from animal studies to human trial. *BioMetals*. 23:399–409. doi:10.1007/s10534-010-9331-3.

Tsuda, H., K. Sekine, K. Fujita, and M. Iigo. 2002. Cancer prevention by bovine lactoferrin and underlying mechanisms — a review of experimental and clinical studies. *Biochem. Cell Biol.* 80:131–136. doi:10.1139/o01-239.

Tung, Y.-T., H.-L. Chen, C.-C. Yen, P.-Y. Lee, H.-C. Tsai, M.-F. Lin, and C.-M. Chen. 2013. Bovine lactoferrin inhibits lung cancer growth through suppression of both inflammation and expression of vascular endothelial growth factor. *J. Dairy Sci.* 96:2095–106. doi:10.3168/jds.2012-6153.

Viejo-Díaz, M., M.T. Andrés, and J.F. Fierro. 2004. Modulation of in vitro fungicidal activity of human lactoferrin against *Candida albicans* by extracellular cation concentration and target cell metabolic activity. *Antimicrob. Agents Chemother.* 48:1242–8. doi:10.1128/AAC.48.4.1242.

Viejo-Díaz, M., M.T. Andrés, J. Pérez-Gil, M. Sánchez, and J.F. Fierro. 2003. Potassium efflux induced by a new lactoferrin-derived peptide mimicking the effect of native human lactoferrin on the bacterial cytoplasmic membrane. *Biochem.* 68:217–27.

Vishvakarma, N.K., and S.M. Singh. 2011. Mechanisms of tumor growth retardation by modulation of pH regulation in the tumor-microenvironment of a murine T cell lymphoma. *Biomed. Pharmacother.* 65:27–39. doi:10.1016/j.biopha.2010.06.012.

Vogel, H.J. 2012. Lactoferrin, a bird's eye view. *Biochem. Cell Biol.* 90:233–44. doi:10.1139/o2012-016.

Wang, J., Q. Li, Y. Ou, Z. Han, K. Li, P. Wang, and S. Zhou. 2011. Inhibition of tumor growth by recombinant adenovirus containing human lactoferrin through inducing tumor cell apoptosis in mice bearing EMT6 breast cancer. *Arch. Pharm. Res.* 34:987–95. doi:10.1007/s12272-011-0616-z.

Wang, J., Q. Li, Y. Ou, K. Li, Z. Han, P. Wang, and S. Zhou. 2012a. Recombination adenovirus-mediated human lactoferrin cDNA inhibits the growth of human MCF-7 breast cancer cells. *J. Pharm. Pharmacol.* 64:457–63. doi:10.1111/j.2042-7158.2011.01410.x.

Wang, J., X. Shi, H. Wang, X. Xia, and K. Wang. 2012b. Effects of Esterified Lactoferrin and Lactoferrin on Control of Postharvest Blue Mold of Apple Fruit and Their Possible Mechanisms of Action. *J. Agric. Food Chem.* 60:6432–6438.

Wang, L., Z. Fan, J. Zhang, Y. Changyi, C. Huang, Y. Gu, Z. Xu, Z. Tang, W. Lu, X. Wei, and C. Li. 2014. Evaluating tumor metastatic potential by imaging intratumoral acidosis via pH-activatable near-infrared fluorescent probe. *Int. J. Cancer*. doi:10.1002/ijc.29153.

Wang, W., M. Iigo, J. Sato, K. Sekine, and I. Adachi. 2000. Activation of Intestinal Mucosal Immunity in Tumor-bearing Mice by Lactoferrin. *Jpn. J. Cancer Res.* 91:1022–1027.

Ward, P.P., E. Paz, and O.M. Conneely. 2005. Multifunctional roles of lactoferrin: a critical overview. *Cell. Mol. Life Sci.* 62:2540–8. doi:10.1007/s00018-005-5369-8.

Welsh, K.J., S. Hwang, S. Boyd, M.L. Kruzel, R.L. Hunter, and J.K. Actor. 2011. Influence of oral lactoferrin on *Mycobacterium tuberculosis* induced immunopathology. *Tuberc.* Suppl 1:S105–13. doi:10.1016/j.tube.2011.10.019.Influence.

WHO. 2014. World Health Organisation. *http://www.who.int/gho/ncd/mortality_morbidity/cancer/en/index.html*. visited on 13 November 2014.

Wojtkowiak, J.W., D. Verduzco, K.J. Schramm, and R.J. Gillies. 2011. Drug resistance and cellular adaptation to tumor acidic pH microenvironment. *Mol Pharm.* 8:2032–2038. doi:10.1021/mp200292c.Drug.

Wolf, J.S., G. Li, A. Varadhachary, K. Petrak, M. Schneyer, D. Li, J. Ongkasuwan, X. Zhang, R.J. Taylor, S.E. Strome, and B.W. O'Malley. 2007. Oral lactoferrin results in T cell-dependent tumor inhibition of head and neck squamous cell carcinoma in vivo. *Clin. Cancer Res.* 13:1601–10. doi:10.1158/1078-0432.CCR-06-2008.

Wu, Y., W. Zhang, J. Li, and Y. Zhang. 2013. Optical imaging of tumor microenvironment. *Am. J. Nucl. Med. Mol. Imaging*. 3:1–15.

Xiao, Y., C.L. Monitto, K.M. Minhas, and D. Sidransky. 2004. Lactoferrin Down-Regulates G 1 Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells Lactoferrin Down-Regulates G 1 Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells. *Clin Cancer Res*. 10:8683–8686.

Xu, M., M. Xia, X.-X. Li, W.-Q. Han, K.M. Boini, F. Zhang, Y. Zhang, J.K. Ritter, and P.-L. Li. 2012. Requirement of translocated lysosomal V1 H(+)-ATPase for activation of membrane acid sphingomyelinase and raft clustering in coronary endothelial cells. *Mol. Biol. Cell*. 23:1546–57. doi:10.1091/mbc.E11-09-0821.

Xu, X.X., H.R. Jiang, H.B. Li, T.N. Zhang, Q. Zhou, and N. Liu. 2010. Apoptosis of stomach cancer cell SGC-7901 and regulation of Akt signaling way induced by bovine lactoferrin. *J. Dairy Sci.* 93:2344–50. doi:10.3168/jds.2009-2926.

Xu, Y.Y., Y.H. Samaranayake, L.P. Samaranayake, and H. Nikawa. 1999. *In vitro* susceptibility of *Candida* species to lactoferrin. *Med. Mycol.* 37:35–41.

Yamano, E., M. Miyauchi, H. Furusyo, A. Kawazoe, A. Ishikado, T. Makino, K. Tanne, E. Tanaka, and T. Takata. 2010. Inhibitory effects of orally administrated liposomal bovine lactoferrin on the LPS-induced osteoclastogenesis. *Lab. Invest.* 90:1236–46. doi:10.1038/labinvest.2010.80.

Yen, C.-C., C.-J. Shen, W.-H. Hsu, Y.-H. Chang, H.-T. Lin, H.-L. Chen, and C.-M. Chen. 2011. Lactoferrin: an iron-binding antimicrobial protein against Escherichia coli infection. *Biometals*. 24:585–94. doi:10.1007/s10534-011-9423-8.

Yoo, Y.-C., R. Watanabe, Y. Koike, M. Mitobe, K. Shimazaki, S. Watanabe, and I. Azuma. 1997. Apoptosis in Human Leukemic Cells Induced by Lactoferricin, a Bovine Milk Protein-Derived Peptide: Involvement of Reactive Oxygen Species. *Biochem. Biophys. Res. Commun.* 237:624–628. doi:10.1006/bbrc.1997.7199.

Yoshinaka, K., H. Kumanogoh, S. Nakamura, and S. Maekawa. 2004. Identification of V-ATPase as a major component in the raft fraction prepared from the synaptic plasma membrane and the synaptic vesicle of rat brain. *Neurosci. Lett.* 363:168–72. doi:10.1016/j.neulet.2004.04.002.

Zarember, K.A., J.A. Sugui, Y.C. Chang, K.J. Kwon-Chung, and J.I. Gallin. 2007. Human Polymorphonuclear Leukocytes Inhibit *Aspergillus fumigatus* Conidial Growth by Lactoferrin-Mediated Iron Depletion. *J Immunol*. 178:6367–6373.

Zhang, X., Y. Lin, and R.J. Gillies. 2010. Tumor pH and its measurement. *J. Nucl. Med.* 51:1167–70. doi:10.2967/jnumed.109.068981.

Zhang, Y., A. Nicolau, C.F. Lima, and L.R. Rodrigues. 2014a. Bovine Lactoferrin Induces Cell Cycle Arrest and Inhibits Mtor Signaling in Breast Cancer Cells. *Nutr. Cancer*. 0:1–15. doi:10.1080/01635581.2014.956260.

Zhang, Y., C.F. Lima, and L.R. Rodrigues. 2014b. In vitro evaluation of bovine lactoferrin potential as an anticancer agent. *Int. Dairy J.* 40:6–15. doi:10.1016/j.idairyj.2014.08.016.

Zhang, W., S. Fan, G. Zou, L. Shi, Z. Zeng, J. Ma, Y. Zhou, X. Li, X. Zhang, X. Li, M. Tan, W. Xiong, and G. Li. 2014c. Lactotransferrin could be a novel independent molecular prognosticator of nasopharyngeal carcinoma. *Tumour Biol.* doi:10.1007/s13277-014-2650-1.

Zhou, X., W. Xu, X. Tang, L. Luo, J. Tu, C. Zhang, X. Xu, Q. Wu, and W. Pan. 2014. Fecal lactoferrin in discriminating inflammatory bowel disease from Irritable bowel syndrome: a diagnostic meta-analysis. *BMC Gastroenterol.* 14:121. doi:10.1186/1471-230X-14-121.

Zhou, Y., Z. Zeng, W. Zhang, W. Xiong, M. Wu, Y. Tan, W. Yi, L. Xiao, X. Li, C. Huang, L. Cao, K. Tang, X. Li, S. Shen, and G. Li. 2008. Lactotransferrin: a candidate tumor suppressor-Deficient expression in human nasopharyngeal carcinoma and inhibition of NPC cell proliferation by modulating the mitogen-activated protein kinase pathway. *Int. J. Cancer*. 123:2065–72. doi:10.1002/ijc.23727.

CHAPTER II

LACTOFERRIN AS A NOVEL PROTON PUMP INHIBITOR TARGETING THE PLASMA MEMBRANE V-ATPASE OF CANCER CELL: A MOLECULAR MECHANISM UNDERLYING ITS ANTI-TUMORAL ACTIVITY

Part of the results presented in this chapter were obtained in collaboration with Marília Gonçalves and Luís Loureiro under the scope of their master thesis and will be submitted for publication:

Pereira, C., Gonçalves, M; Loureiro, L.; Castro, L.; Gerós, H.; Rodrigues, L. R. and Côrte-Real, M. Lactoferrin as a novel proton pump inhibitor targeting the plasma membrane V-ATPase of the cancer cell: a mechanism underlying its anti-tumoral activity.

II.1. MATERIALS AND METHODS

II.1.1. Chemicals and solutions

Bovine lactoferrin (bLf) was obtained from DMV (Veghel, The Netherlands). The protein purity is about 80% with 3.5% moisture and 21% iron-saturated according to the manufacturer. It was dissolved in phosphate buffered saline (PBS) (1.37 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to obtain the different concentrations used throughout this work.

Concanamycin A (ConcA), phenolsulfonephthaleine (Phenol Red), sulforhodamine B (SRB), propidium iodide (PI), RNase, paraformaldehyde (PFA), filipin and methyl-β-ciclodextrin (MβCD) were obtained from Sigma-Aldrich. Bafilomycin A1 (BafA1) was purchased from Acros Organics. BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester), ACMA (9-amino-6-chloro-2-methoxyacridine), Alexa fluor 488-Phalloidin and Alexa fluor 647 were obtained from Molecular Probes. Vectashield mounting medium was purchased from Vector Laboratories. The anti-V-ATPase antibody was purchased from Millipore-Merck, anti-Tom20 from Santa Cruz Biotechnology and anti-GAPDH from Hytest; the secondary antibodies Peroxidase-AffiniPure goat anti-rabbit IgG and goat anti-mouse IgG were acquired from Jackson ImmunoResearch.

II.1.2. Cell lines and culture conditions

Human breast cancer cell lines T-47D (HTB-113; ATCC) and Hs 578T (HTB-126; ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. MCF-10-2A (CRL-10781; ATCC) cells were grown in DMEM-F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone. All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For all the experiments cells were seeded in 6-well or 96-well plates at a concentration of 2×10^5 cells/ml.

II.1.3. Assessment of cell proliferation by Sulforhodamine B assay

Breast cells were seeded in 6-well plates and incubated with different bLf concentrations (50, 125 and 175 µM) for 24 and 48 h, after which they were fixed 90 min at -20 °C in ice-cold 1% acetic acid in methanol and then incubated with 0.5% (w/v) SRB in 1% acetic acid for 90 min at 37 °C. After washing with 1% acetic acid and drying, protein-bound SRB was dissolved in 10 mM Tris for 10 min at room temperature (RT). A sample of each condition was transferred to a 96-well plate and absorbance was read at 540 nm in a microplate reader (SpectraMax 340PC Molecular Devices). Results were normalized to the untreated cells, which were considered as 100% cell proliferation.

II.1.4. Cell cycle analysis

Cells were seeded in 6-well plates and treated with different bLf concentrations (50, 125 and 175 µM) for 24 and 48 h. After harvesting with a cell scrapper, cells were fixed in ice-cold absolute ethanol. Next, they were incubated with RNAse (in sodium citrate (1% w/v)) for 15 min at 37 °C, followed by PI (in sodium citrate (1% w/v)) for 30 min at RT. Cell cycle distribution and appearance of a sub-G0/G1 population were determined by flow cytometry.

II.1.5. Extracellular pH measurement

The extracellular pH (pHe) was estimated using Phenol Red absorbance as described elsewhere (Montcourrier et al., 1997) with some modifications. Cells were seeded in 96-well plates and allowed to adhere for 24 h, after which they were washed with HBSS - Hank's Balanced Salt Solution (138 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.35 mM Na_2HPO_4, 2 mM L-glutamine) containing 0.03 mM Phenol Red and then incubated for a total of 120 min at 37 °C with humidified atmosphere in a Biotech Synergy HT microplate reader. The absorbance ratio at 562/450 nm was monitored every 15 min. The pH values were calculated from the equation $pH = pK'_a + \log [(R - R_{min})/(R_{max} - R)]$, where R is the absorbance ratio, Rmax and Rmin the limiting values of R (from the calibration curve), and pK'_a the negative log of the dissociation constant of Phenol Red (pK_a Phenol Red = 7.9). When cells were incubated with bLf or ConcA prior

to pHe measurement, they were seeded in 6-well plates and Phenol Red absorbance was only monitored at 0 and 120 min after incubation in HBSS solution. The variation in pHe was calculated from these two values. All the results were expressed with reference to DNA quantified using a nanodrop after cell lysis with a solution containing 1% SDS and 0.2 M NaOH. For the calibration curve, HBSS solutions containing 0.03 mM Phenol Red and 50 mM HEPES in a pH range from 6.4 to 8.3 were prepared and the pH was also monitored by absorbance at 562/450 nm. The calibration curve (Fig. IV.1) shows a good correlation between the estimated and the effective pH, attesting the reliability of this method.

II.1.6. Intracellular pH measurement

Measurement of intracellular pH (pHi) was performed with the pH-sensitive probe BCECF-AM. Hs 578T cells were seeded in 6-well plates and incubated with 175 µM bLf for 1 and 24 h. For each incubation time, cells were tripsinized and loaded with 20 µM BCECF-AM at 37 °C for 30 min. After centrifugation at 2000 rpm during 5 min to remove the medium, they were washed in HBSS pH 7.0 and analysed by flow cytometry. The variations in pHi were estimated from the ratio of green/red fluorescence intensities (FL1/FL3).

II.1.7. Flow cytometric analysis

Flow cytometry analysis was performed in an Epics® XLTM (BeckmanCoulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass filter. Red fluorescence was collected through a 560 nm short-pass dichroic, a 640 nm long-pass, and another 670 nm long-pass filter. For each sample 20 000 events were evaluated and data were analysed using the Flowing Software 2.0 for BCECF-AM experiments and FlowJo 7.6 for cell cycle analysis.

II.1.8. Immunofluorescence and confocal microscopy

For immunofluorescence experiments, cells were seeded in 6-well plates containing glass coverslips. After 24 h, cells were fixed with 4% paraformaldehyde (PFA) for 40 min. After rinsing with PBS 1×, cells were incubated with ammonium chloride 50 mM during 10 min, rinsed again and permeabilized with PBS-SDS 0.1% for 10 min. Cells were then blocked in PBS-BSA 3% for 20 min and washed. Following this procedure, cells were incubated with primary antibody anti-ductin, which detects subunit *c'* of V-ATPase, in PBS-BSA 0.1% overnight at 4 °C in a humidified chamber. Subsequently, cells were washed with PBS-BSA 0.1% and then labelled with Alexa fluor 647 (1:200) for 1 h in the dark. After rinsing, cells were finally incubated with Alexa fluor 488-Phalloidin to stain F-actin and delineate the cell cytoskeleton for 1 h in the dark. After mounting the coverslip in Vectashield mounting medium (Vector Laboratories), samples were maintained at -20 °C until visualization. Images were acquired in a sequential mode by Confocal Scanning Laser Microscope OLYMPUS BX61/FLUOVIEW1000, using a 60× oil immersion objective and the specific filter settings for Alexa fluor 488 and 647. For all the images acquired a negative control corresponding to cells labelled only with the secondary antibody Alexa fluor 647 (Fig. IV.2) was performed in order to attest the specific staining of V-ATPase subunit *c*' when the primary antibody was used.

II.1.9. Filipin staining

Cells were seeded in 6-well plates containing glass coverslips and allowed to attach for 24 h. Subsequently, the cells were incubated with fresh medium or fresh medium containing methyl-β-ciclodextrin or bLf. After 2 or 24 h of incubation, respectively, cells were fixed with 4% PFA for 40 min. Next, PFA was quenched by incubation of fixed cells with a 1.5 mg glycine/ml PBS solution for 15 min at RT. Cells were then stained with 0.01 mg/mL filipin dissolved in PBS 1× supplemented with 0.5% BSA for 2 h at room temperature in the dark. Finally, cells were washed with PBS 1×, mounted on slides with the anti-fading agent Vectashield (Vector Laboratories) to overcome the instability of this dye, and visualized in a fluorescence microscope (Leica DM 5000B, Leica Microsystems).

II.1.10. Crude membrane fraction isolation

Crude membrane fractions containing lysosomes were obtained with the Focus SubCell kit from GBiosciences. Among both cancer cell lines, only the T-47D yielded enough amount of membrane vesicles to measure the V-ATPase activity by spectrofluorimetry. Briefly, after harvesting and lysing the cells (5×10^7 cells/ml), the extracts were submitted to a series of differential centrifugations that allowed the removal of nuclear, mitochondrial and cytosolic fractions, being the resulting fraction enriched in cellular membranes.

II.1.11. Rat liver lysosomes isolation

Sprague-Dawley female rats (225 gr; Charles-River, Spain) were maintained in appropriate cages in a temperature-controlled room (22-23 °C) on 12 h light: 12 h dark cycle, and fed with commercial solid food. All procedures were performed according to the Portuguese law for animal welfare. The rats were sacrificed by decapitation and their livers collected and immediately kept in a cold buffer (0.25 M sucrose in 10 mM Tris-HCl, pH 7.4). Then, the liver was minced and homogenized in a Potter-Elvehjem. After centrifugation at 12000 g for 10 min, the pellet was discarded and $CaCl_2$ solution (0.08 M $CaCl_2$ in 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4) was added to the supernatant at a final concentration of 8 mM to cause mitochondrial swelling. The sample was then centrifuged at 25000 g for 15 min. The pellet was resuspended in 30 mL of KCl buffer (150 mM KCl in 10 mM Tris-HCl, pH 7.4) and centrifuged again at 25000 g for 15 min. The resulting pellet was finally resuspended in 2 mL of KCl buffer and kept on ice.

II.1.12. Protein quantification

Protein concentration was determined by the Lowry method (Lowry et al., 1951), using BSA (5 µg/µL - 25 µg/µL) to build a standard curve. Briefly, solution A (NaK Tartrate, $CuSO_4$, NaOH, Na_2CO_3) was added to each sample plus 5% SDS and incubated for 5 min at 37 °C. Next, Folin reagent was added and incubated in the same conditions. Absorbance at 700 nm was monitored and protein concentration was estimated in relation to the standard curve.

II.1.13. Measurement of proton pumping activity of V-ATPase

Proton pumping activity of V-ATPase in isolated lysosomes/crude membranes was evaluated by the measurement of the ACMA fluorescence quenching in a Perkin-Elmer LS-5B spectrofluorimeter. The excitation/emission wavelengths were set to 415 nm and 485 nm, respectively. The reaction medium contained 1 mM MOPS (3-(N-morpholino)propanesulfonic acid)-Tris pH 7.2, 100 mM KCl, 2 µM ACMA, 12.5 mM $MgCl_2$, and 200 µg of lysosomes/crude membranes in a final volume of 2 ml. The reaction was initiated by adding 0.5 mM ATP and the rate of initial fluorescence quenching was recorded. For the analysis of the instantaneous effect, 10 nM ConcA, 20 nM BafA1 and different concentrations of bLf (0.05, 0.5, 1.0, 5.0, 10.0 µM) were directly added to the assay medium after stabilization of the reaction started by ATP. The other experiment was performed by pre-incubation for different times (0, 30, 60 and 120 min) with 1 µM bLf. The ACMA fluorescence quenching was considered the V-ATPase H^+-transport activity [$\Delta\%F$ min^{-1} $(mg\ prot)^{-1}$] and the fluorescence quenching recovery as the inhibition of this activity. The results were analysed using the GraphPad Prism Software.

II.1.14. Measurement of hydrolytic activity of V-ATPase

The rate of ATP hydrolysis was determined by measuring the release of inorganic phosphate (Pi) according to the procedure described by Vera-Estrella et al., 1994 with some modifications. Isolated lysosomes (50 µg protein) were mixed with 300 µL of 3 mM ATP (or different ATP concentrations for the kinetics analysis), 0.02% Triton X-100, 50 mM KCl, 1 mM sodium molybdate, 6 mM $MgSO_4$ in 30 mM Tris pH 8, plus the desired treatment, and incubated for 30 min at 37 °C, with slow agitation (80 rpm). The reaction was stopped by adding 500 µL of a cold solution containing 10% trichloroacetic acid (TCA) and 4% perchloric acid. Samples were kept on ice for 5 min, centrifuged for 3 min at 2400 g, and 500 µL of the supernatant were mixed with 1.3 mL of Ames solution composed of 1 volume of 10% ascorbic acid mixed with 6 volumes of 21.4 mM ammonium molybdate and 53.6 mM of H_2SO_4. After 15 min at RT in the dark, absorbance was read at 820 nm using a blank control performed without protein and NaH_2PO_4 as standard to establish a calibration curve. For all the experiments, a control sample without protein was prepared to subtract the spontaneous hydrolysis induced by the

compounds. The K_m values of the V-ATPase hydrolytic activity in the absence and presence of bLf were estimated by fitting the data points to a Michaelis-Menten type kinetics using the GraphPad Prism Software.

II.1.15. Western blot analysis

Protein samples (50 µg) of isolated lysosomal fractions or cell homogenates were separated by sodium dodecyl sulfate 15% polyacrylamide gel electrophoresis and transferred onto PVDF (polyvinylidene difluoride) membranes. Next, membranes were blocked in 5% non-fat milk in PBS-Tween 0.1% solution for 3 h with agitation to avoid non-specific interactions. Membranes were then incubated overnight at 4 °C with the primary antibodies, namely rabbit polyclonal anti-V-ATPase subunit *c′* (Millipore-Merk), rabbit polyclonal anti-Tom20 (Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH (Hytest), followed by incubation with secondary antibodies Peroxidase-AffiniPure goat anti-rabbit IgG or goat anti-mouse IgG (1:2000; Jackson ImmunoResearch). Chemiluminescence detection was performed using the ECL detection system (Millipore-Merck) and a Chemi-Doc XRS system (BioRad).

II.1.16. Statistical Analysis

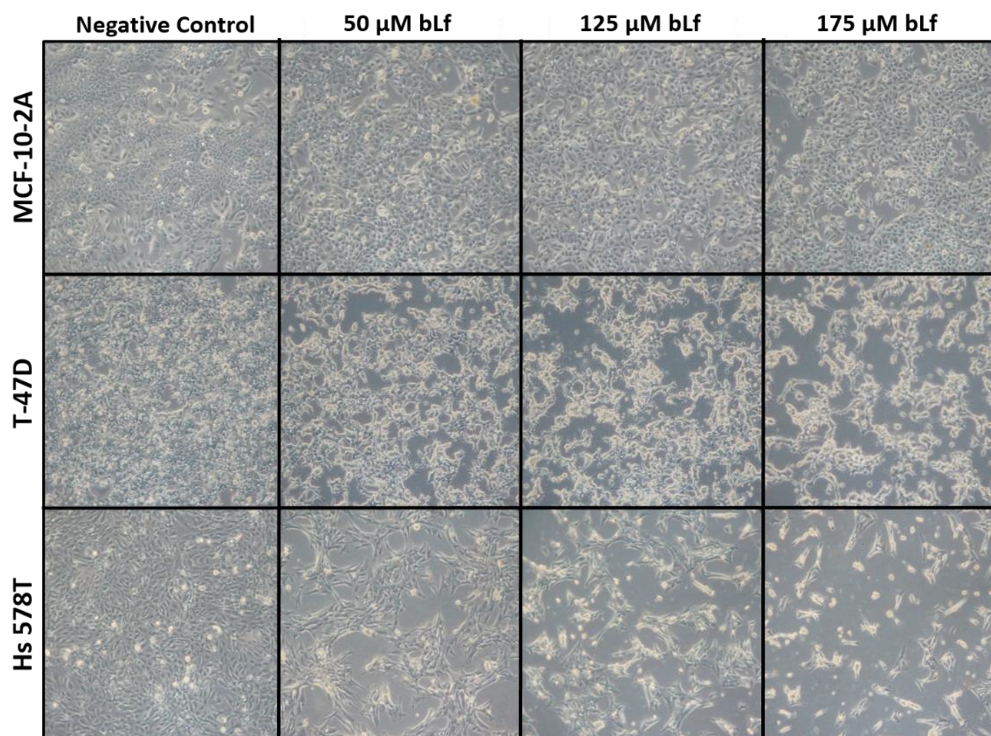
Data are expressed as means ± S.E.M. of at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-test (assumes that all the tests are independent from each other) using the GraphPad Prism version 5.0.

II.2. RESULTS

II.2.1 Differential extracellular acidification capacity and V-ATPase localization determine breast cell lines susceptibility to bovine lactoferrin

In order to get insight into the molecular mechanism underlying the bLf anti-tumoral activity, our election model was breast cancer as there are only few reports concerning the effect of this protein. We used three breast cell lines displaying the molecular backgrounds that suit our study, namely Hs 578T and T-47D, an invasive and a non-invasive breast cancer cell lines, respectively, and also the non-tumorigenic cell line MCF-10-2A to serve as a control of normal epithelial breast cells. Cells were incubated with three different bLf concentrations (50, 125 and 175 µM) for 24 and 48 h. The cell proliferation, assessed by the SRB assay, was not significantly affected after a 24 h treatment with bLf. However, after 48 h, a 50% decrease in the proliferation of the invasive cell line Hs 578T was found for the highest bLf concentration tested (175 µM), in contrast with the two other cell lines (Fig. II.1 A, B).

(A)



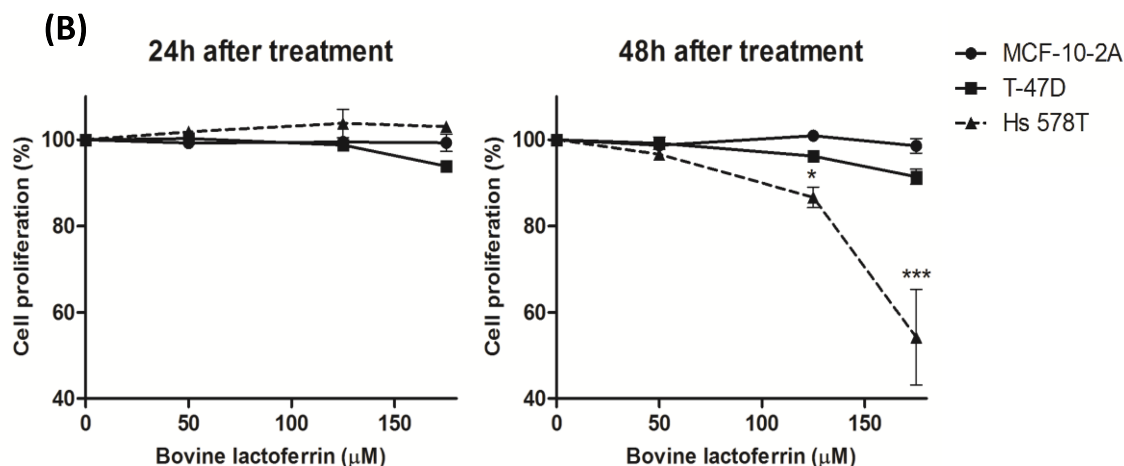


Figure II.1: Analysis of the susceptibility of breast cell lines to bLf. (A) Representative images of the effects of bLf (50, 125 and 175 μM) 48 h treatment on Hs 578T, T-47D and MCF-10-2A cell lines (phase contrast 10×). **(B)** Cell proliferation analysis by the SRB assay in the same breast cells treated with increasing doses of bLf for 24 and 48 h. Values represent mean ± S.E.M. of at least three independent experiments, *P<0.05; ***P<0.001 compared with MCF-10-2A cells.

The observed decrease in cell proliferation might be due to an effect of bLf in the cell cycle progression and/or induction of apoptosis as reported in other studies (Xiao et al., 2004; Lee et al., 2009). Therefore, we analysed the cell cycle progression, as well as the presence of an apoptotic sub-population in cell suspensions through the assessment of cellular DNA content by flow cytometry. The appearance of a sub-G0/G1 population is indicative of DNA degradation and has been used as an apoptotic marker. Although for the bLf-treated Hs 578T cells there seems to be an arrest of cells in the S phase, the overall results revealed that bLf had no statistically significant effects on the distribution of cells among the cell cycle phases (G1, S and G2/M) (Fig. II.2 A). Fig. II.2 B illustrates the representative DNA content histograms of control and bLf-treated cells, in which two peaks corresponding to cells in G1 and G2/M phases of cell cycle are evident. For the untreated samples, almost no cells with a hypodiploid DNA content were found, while for the cancer cells treated with bLf this frequency greatly increased, having particular significance for the Hs 578T cell line. The comparison of the percentage of the sub-G0/G1 population in cells treated with increasing doses of bLf after a 24 and 48 h exposure indicates that this protein induces a dose-dependent increase in the sub-G0/G1 population in the two breast cancer cell lines, evidencing again their distinct susceptibilities to bLf. Indeed, the MCF-10-2A, T-47D and Hs 578T percentage of sub-

G0/G1 phase cells after 48 h incubation with 175 µM bLf was approximately 2%, 25% and 50%, respectively (Fig. II.2 C), which is in accordance with the observed impact on cell proliferation. Taken together, these results suggest that bLf inhibits cell proliferation associated with induction of apoptosis in the highly metastatic cell line Hs 578T and to a lesser extent in the poorly invasive cell line T-47D. On the other hand, bLf presents a negligible effect in the non-tumorigenic breast cell line MCF-10-2A, which indicates a specific targeting of this protein to the cancer cells, in particular to the invasive cells, and strengthens the bLf potential as an anticancer therapeutic agent without side effects to the normal cells.

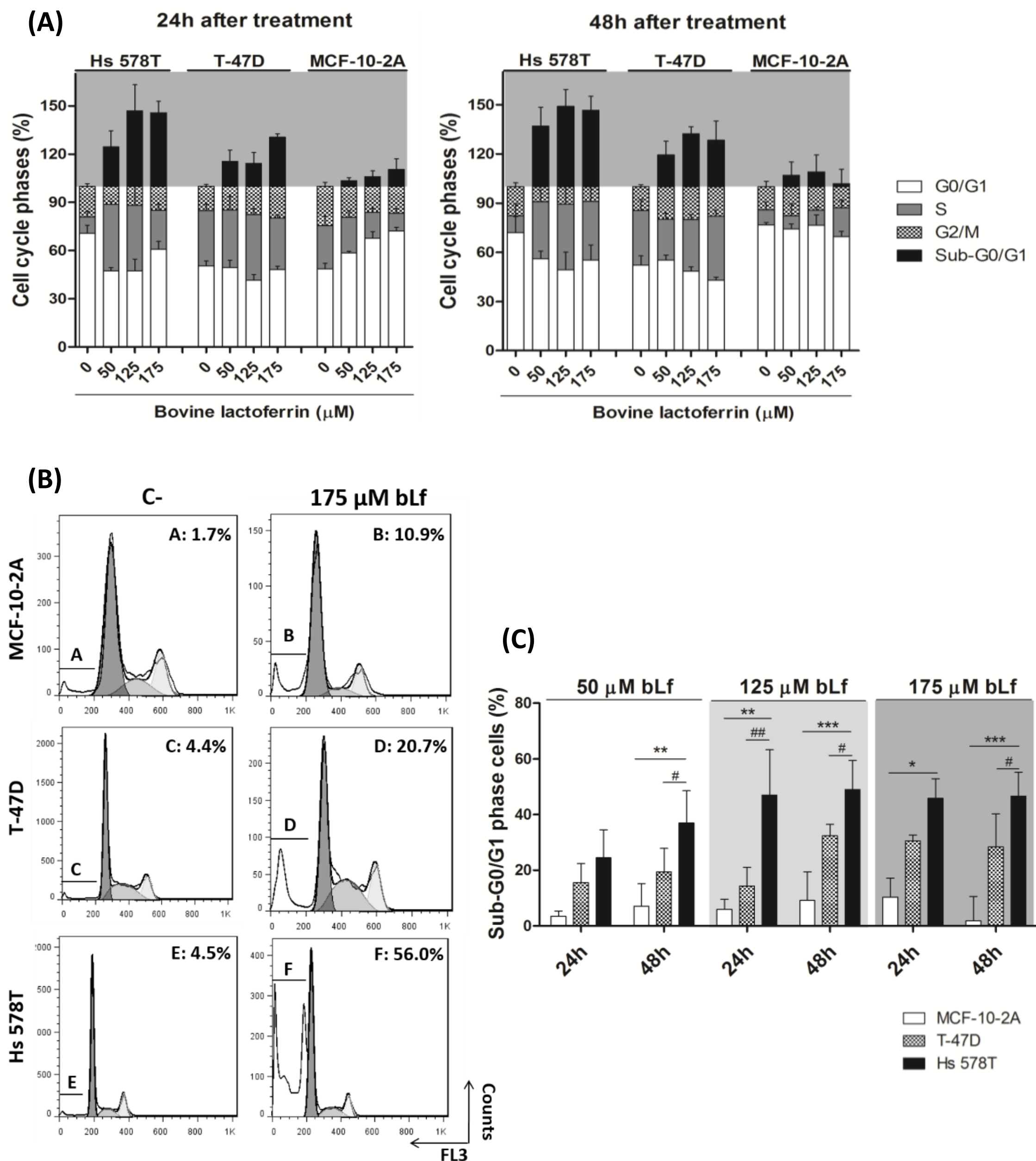


Figure II.2: Effect of bLf on the cell cycle distribution and apoptosis of breast cell lines. (A) Analysis of the distribution of cell cycle phases and of the appearance of an apoptotic sub-G0/G1 population by flow cytometry of breast cells labelled with PI after treatment with bLf. Cells were incubated with fresh medium in the absence or presence of increasing doses of bLf (50, 125 and 175 µM) for 24 and 48 h. **(B)** Representative histograms of control breast cells and of cells incubated with 175 µM bLf for 48 h. Percentage of sub-G0/G1 cells is indicated for each condition (A-F). **(C)** Comparison of the percentage of sub-G0/G1 cells after treatment with bLf for 24 and 48 h in the three breast cell lines. Values represent mean ± S.E.M. of at least three independent experiments, *P<0.05; **P<0.01; ***P<0.001 percentage of sub-G0/G1 population of Hs 578T cells compared to MCF-10-2A cells; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 percentage of sub-G0/G1 population of Hs 578T cells compared to T-47D cells.

Since invasive tumor cells are known to exhibit a higher extracellular acidification ability, associated with a re-localization of V-ATPase to the plasma membrane (Sennoune et al., 2004), and Lf was previously suggested to interact with H^+ pumps in bacteria and fungi (Andrés and Fierro 2010; Wang et al. 2012a), we then questioned whether the different cell line susceptibilities to bLf could be attributed to a differential cellular localization of V-ATPase in the three cell lines. To address this hypothesis we first monitored the cell lines extracellular acidification capacity and found that the cancer cells display a higher extracellular acidification capacity than the non-tumorigenic ones. Additionally, the invasive cells exhibited a two- and a three-fold decrease in the extracellular pH when compared with T-47D and MCF-10-2A cells, respectively (Fig. II.3 A). In conclusion, these results support a direct relation between the extracellular acidification capacity and the sensitivity of the three cell lines to bLf.

Subsequently, we sought whether the distinct extracellular acidification ability could be connected to a different localization of V-ATPase in the cell lines under study. Thus, we used two antibodies, one against subunit *c'* of V-ATPase and other against F-actin to delineate the cytoskeleton and the cell boundaries. Immunofluorescence showed variations in the localization of V-ATPase in the three cell lines (Fig. II.3 B). An intracellular distribution was visualized in all cell lines which is in agreement with the known localization of this proton pump in the intracellular compartments like lysosomes (Jefferies et al., 2009). Notably, we confirmed a plasma membrane distribution in various optical sections of the Hs 578T invasive cells (head arrows). This observation supports the conclusion that most of the V-ATPase cell content is exerting its function at the plasma membrane and has a major contribution to the acidification of the

extracellular milieu of invasive cells. Regarding the other two cell lines studied, no membrane localization of V-ATPase was detected in the non-tumorigenic cells and almost no signal could be identified at the plasma membrane of the T-47D cells. Indeed, these cell lines exhibit a predominantly intracellular localization of V-ATPase.

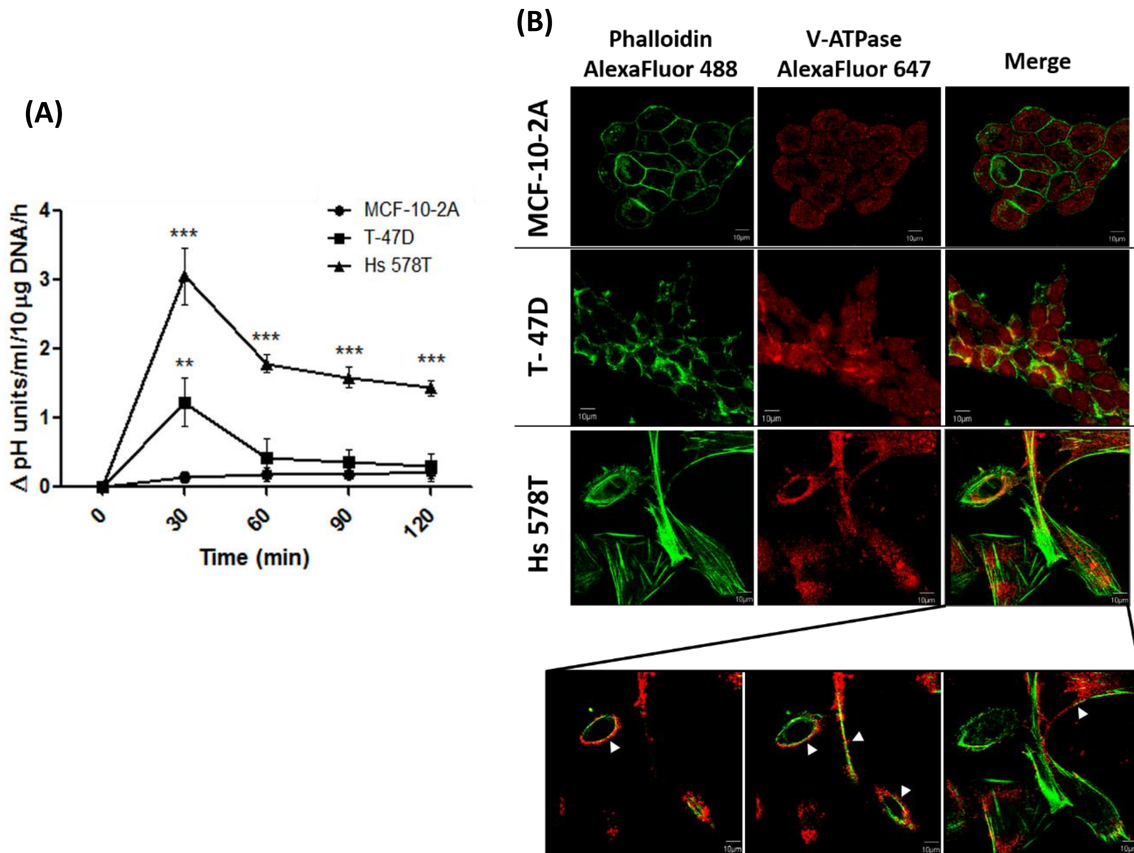


Figure II.3: Analysis of the extracellular acidification capacity and cellular distribution of V-ATPase on breast cells. (A) Extracellular acidification induced by MCF-10-2A, T-47D and Hs 578T breast cells expressed as the decrease in the extracellular pH. pH changes were monitored by phenol red absorbance at 562/450 nm after 0, 30, 60, 90 and 120 min of incubation with HBSS buffer containing 1 mM glucose, and calculated using the equation $pH = pK'_a + \log[(R - R_{min})/(R_{max} - R)]$ as described in Materials and Methods. Results are expressed in pH units change per ml of HBSS buffer per h, and per 10 µg DNA. Values represent the mean ± S.E.M. of three independent experiments, **P<0.01; ***P<0.001 compared with MCF-10-2A cells. **(B)** Representative images of immunofluorescence in the same three breast cells using confocal microscopy. Cells were incubated with primary monoclonal antibody against subunit *c'* of V-ATPase and secondarily labelled with Alexa fluor-647 (red fluorescence). Cytoskeleton was labelled with Alexa fluor 488-Phalloidin (green fluorescence). In MCF-10-2A and T-47D cells, V-ATPase has mainly an intracellular localization, while in Hs 578T cells it is also present at the plasma membrane (co-localization of red and green fluorescence). Insert – Co-localization of F-actin and V-ATPase (yellow dots – head arrows) was observed in various optical sections of Hs 578T cells.

Altogether, our results strongly indicate that the bLf specificity to the metastatic cell line is associated with its higher extracellular acidification capacity and the plasma membrane localization of V-ATPase, thus suggesting that this pump might have a central role in bLf anti-tumoral activity. Concordantly, as the non-tumorigenic cell line does not exhibit such features it is resistant to bLf, which can have important therapeutic implications.

II.2.2. Bovine lactoferrin inhibits the extracellular acidification in invasive breast cancer cells

We next decided to explore the possible involvement of V-ATPase in the bLf-induced apoptosis in the invasive cells. The yielding of a more acidic pHe as a consequence of the V-ATPase re-localization to the plasma membrane, prompted us to study the pHe regulation upon bLf treatment. Therefore, we monitored the extracellular pH in bLf-treated Hs 578T cells and compared it with cells incubated with ConcA, a specific V-ATPase inhibitor. After 2 h, ConcA was able to inhibit the extracellular acidification in about 0.2 pH units, which is in line with its well-known inhibitory effect on H^+ translocation by V-ATPase (Huss et al., 2002), localized at the plasma membrane of these cells. Remarkably, after 6 h, bLf exhibited a similar effect to ConcA showing a pHe increase of about 0.2 pH units when compared to untreated cells (Fig. II.4). Although the differences between the control and treated cells are not statistically significant, it has been proved that pHe variations as low as 0.1 pH units may disrupt important biological processes such as proliferation, migration, invasion and metastasis of tumour cells (Neri and Supuran, 2011). These results strongly suggest that the mechanism by which bLf and ConcA induce alkalinisation of the extracellular milieu are similar. bLf takes more time to induce a similar effect than ConcA probably due to a different targeting of V-ATPase and interaction with cells.

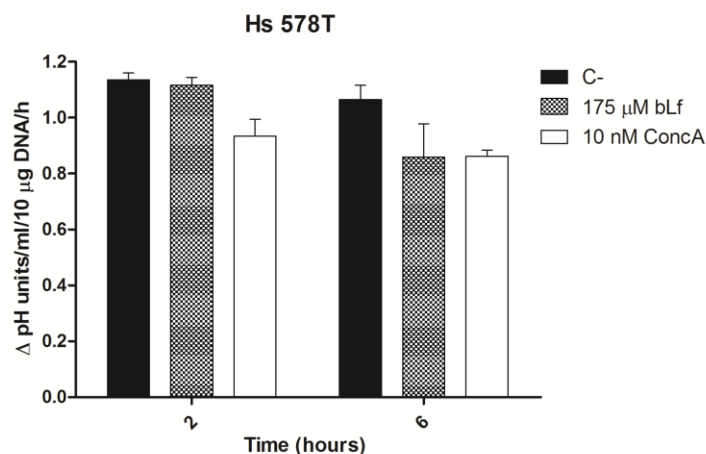


Figure II.4: Measurement of bLf-induced inhibition of extracellular acidification in the invasive breast cancer cell line Hs 578T. pHe measurement after pre-incubation for 2 and 6 h with 10 nM ConcA and 175 µM bLf in Hs 578T breast cells. Alterations in pHe were monitored by phenol red absorbance at 562/450 nm in the pre-treated cells after 0 and 120 min of incubation in HBSS buffer containing 1 mM glucose, and calculated using the equation $pH = pK'_a + \log[(R - R_{min})/(R_{max} - R)]$.

Additionally, our preliminary results indicate that besides extracellular alkalinisation, bLf also causes an intracellular acidification in the Hs 578T cell line (Fig. IV.3), which is more evident after 24 h of incubation with 175 µM bLf. However, a calibration curve using BCECF-AM needs to be established to quantify the pHi decrease caused by bLf and to ascertain if the observed differences are physiologically relevant. This evidence that bLf induces an extracellular alkalinisation associated with an intracellular acidification, is consistent with the hypothetical inhibition of V-ATPase by this protein, which results in H^+ trapping in the cytosol and may underlie the bLf-induced apoptosis in these cells.

II.2.3. Bovine lactoferrin perturbs cholesterol distribution in lipid rafts

As V-ATPase was shown to localize at lipid rafts in metastatic cells, namely in melanoma cells (Baruthio et al., 2008), we speculated whether bLf treatment could alter the lipid raft distribution in breast cells by interacting with V-ATPase. To ascertain this question, rafts were visualized using filipin, a polyene antibiotic with fluorescent properties that binds cholesterol, the major constituent of lipid rafts (Paffett et al., 2011).

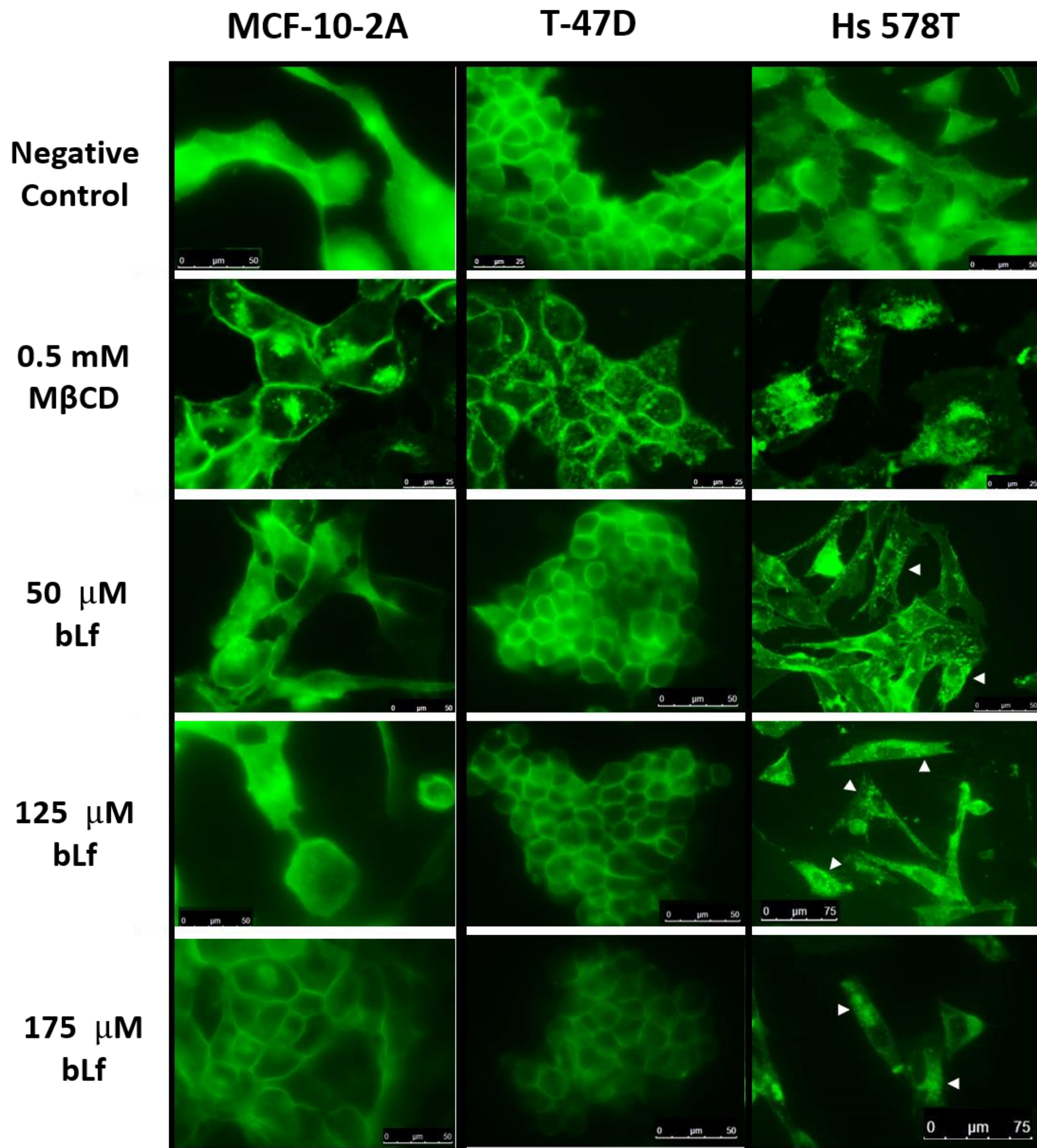


Figure II.5: Distribution of cholesterol-rich lipid rafts in breast cells exposed to bLf. Fluorescence microscopy images of breast cells exposed to increasing doses of bLf (50, 125 and 175 μM) for 48 h or 0.5 mM MβCD for 2 h, and stained with filipin (0.01 mg/ml). bLf alters the distribution of cholesterol-rich domains only in Hs 578T breast cancer cells (head arrows).

The three breast cell lines under study were incubated with increasing concentrations of bLf. In order to confirm the reliability of our approach, we employed the cholesterol-depleting agent methyl-β-ciclodextrin (MβCD) as a positive control of raft disruption. After filipin labelling, in control cells we observed the characteristic plasma membrane staining corresponding to cholesterol mostly localized at lipid rafts, while in MβCD-treated cells this pattern changed as staining of intracellular structures

increased (Fig. II.5). This evidence is in good agreement with previous reports demonstrating that depletion of cholesterol from plasma membrane causes disruption of rafts rendering them non-functional (Li et al. 2006), and inducing delocalization of the associated proteins (Mollinedo et al., 2011). As for the bLf treatment, no effect could be found on MCF-10-2A and T-47D cells, which can be explained by the fact that in these cells the V-ATPase is mainly localized in the intracellular compartments. Conversely, in Hs 578T cells, bLf induced internalization of rafts as demonstrated by intracellular punctate staining (head arrows). These data indicate that cholesterol-rich lipid rafts are re-distributed in the invasive breast cancer cells subjected to bLf treatment, which may give new insights about bLf target in these cells.

II.2.4. Bovine lactoferrin acts as a Proton Pump Inhibitor by targeting V-ATPase

Since our data point to a hypothetical interaction between Lf and V-ATPase, we further analysed its effect on the V-ATPase proton pumping activity. For this purpose, crude membrane fractions containing lysosomes from T-47D cell line were incubated with the pH-sensitive dye ACMA and the fluorescence quenching after addition of ATP, which indicates H^+ transport, was monitored by spectrofluorimetry. Results showed that when bLf was added to the assay medium, the H^+ pumping activity was strongly inhibited, which at the concentration used caused a much greater impact than ConcA and BafA1 (Fig. II.6).

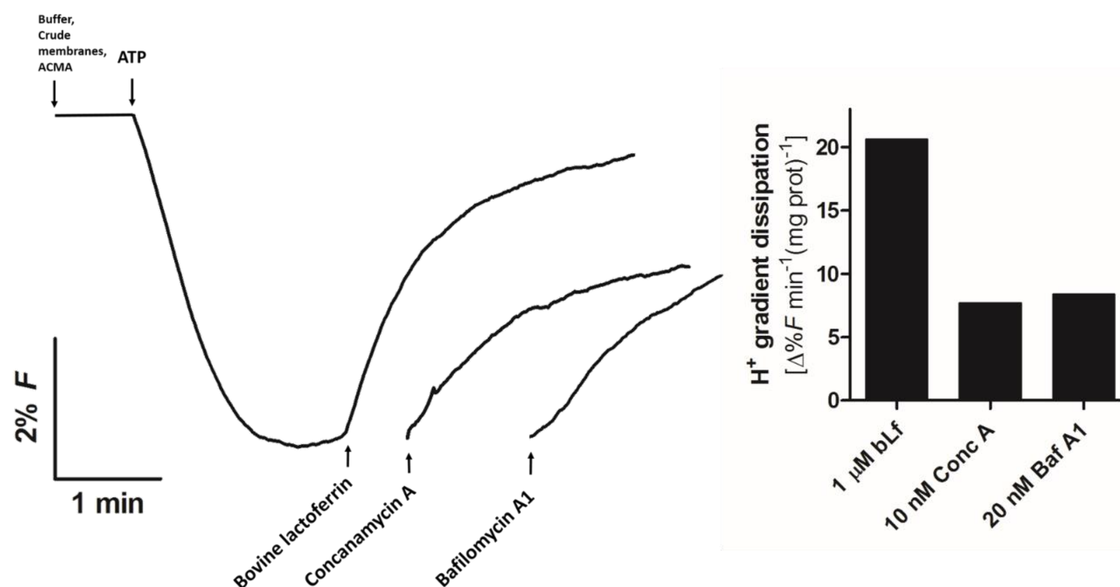


Figure II.6: Measurement of V-ATPase proton pumping activity upon exposure to bLf, ConcA and BafA1 in crude membrane fractions isolated from T-47D cell line. Typical fluorescence signal of the initial velocity of proton pumping by V-ATPase in a crude membrane suspension after adding 0.5 mM ATP and immediate dissipation of the proton gradient by addition of bLf, or the classical V-ATPase inhibitors ConcA and BafA1; quantification of the inhibitory effects by estimating the H^+ gradient dissipation velocity by the respective compounds (plot on the right). These results were obtained in collaboration with M. Gonçalves and L. Loureiro.

In order to ascertain if the substantial impact on H^+ pumping activity observed in T-47D crude membrane suspension was specific for V-ATPase, as other pumps and membrane proteins are present in the crude membrane fractions, we isolated lysosomes from rat liver. Isolated lysosomes have been extensively used to study and characterize lysosomal transport systems like V-ATPase (Arai et al., 1993), and its isolation from rat liver allowed us to obtain a purified and concentrated fraction without mitochondrial or cytosolic contamination (Fig. IV.4). The absence of mitochondria in our fractions is particularly important because it ensures that there is no interference of F-ATPase functioning in the observed results. Addition of either ConcA or BafA1 to these fractions led to the total dissipation of the proton gradient generated after ATP addition, confirming that the intralysosomal acidification was a consequence of the H^+ pumping activity driven by V-ATPase, as these compounds are specific inhibitors of this pump (Fig. II.7 A). The initial velocities of proton pumping by V-ATPase in these fractions were also measured at 0.1-0.7 mM ATP, resulting in a K_m of 0.163 mM (Fig. IV.5). From these results we chose the concentration of 0.5 mM ATP to perform all the experiments as it rendered the maximal proton pumping activity. Consequently, we were able to obtain a clean model of purified and functional lysosomes to study the effect of bLf on V-ATPase activity.

Similarly to what was observed for the crude membrane fraction, bLf was capable of inhibiting proton pumping in the isolated lysosomes although to a lesser extent than the inhibitors (Fig. II.7 A). Then, by estimating the initial velocities of H^+ pumping activity, we performed a kinetic approach in which lysosomes were incubated with 1 μM bLf at different times. It could be concluded that incubation with this protein results in a time-dependent inhibition of the H^+ gradient generation (Fig. II.7 B). Additionally, we also found that bLf induces a dose-dependent immediate H^+ gradient

dissipation (Fig. II.7 C). All these evidences allowed us to propose for the first time that bLf inhibits the H^+ pumping activity of V-ATPase in crude membrane fractions from a non-invasive breast cancer cell line and in a rat liver lysosomal suspension.

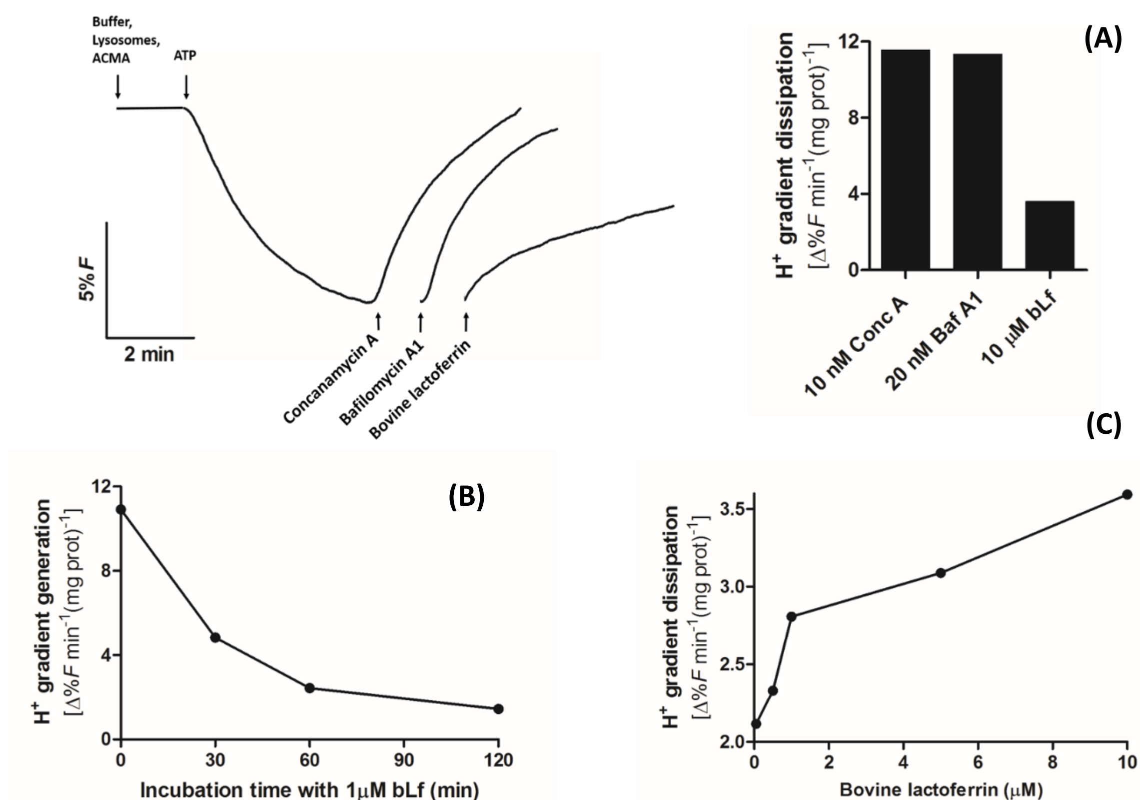


Figure II.7: Measurement of V-ATPase proton pumping activity upon exposure to bLf, ConcA and BafA1 in purified rat liver lysosomes. (A) Typical fluorescence signal of the initial velocity of proton pumping by V-ATPase in a lysosomal suspension after adding 0.5 mM ATP and immediate dissipation of the proton gradient by addition of bLf, ConcA and BafA1; quantification of the respective inhibitory effects by estimating the H^+ gradient dissipation velocity by the respective compounds (plot on the right). **(B)** Time course inhibition of the initial velocity of the V-ATPase proton pumping activity along incubation with bLf (1 µM). **(C)** Quantification of the immediate effect of increasing concentrations of bLf on the proton gradient dissipation. These results were obtained in collaboration with M. Gonçalves and L. Loureiro.

Since V-ATPase couples the energy of ATP hydrolysis to actively transport protons across the lysosomal membrane and bLf inhibits its H^+ pumping activity, we evaluated whether bLf could affect the V-ATPase hydrolytic activity in purified rat liver lysosomes. Similarly to ConcA, bLf also inhibited the V-ATPase hydrolytic activity as the released Pi decreased from 0.18 nmol/min/g in the control lysosomes to 0.08 for ConcA (50 nM) and to 0.06 for bLf (1 µM) (Fig. II.8 A). By performing the appropriate controls

without lysosomes, we also found that bLf itself causes a significant spontaneous ATP hydrolysis that needed to be subtracted from the results in order to obtain a correct measurement of the hydrolytic activity (*data not shown*). Furthermore, the analysis of the V-ATPase hydrolytic activity at 0.0-3.0 mM ATP in the absence and presence of 50 nM bLf followed by Michaelis-Menten kinetics, revealed K_m values of 0.15 and 0.44 mM, respectively, which suggests that bLf may compete with the ATP necessary for the V-ATPase hydrolytic activity (Fig. II.8 B). Whether bLf has a similar inhibition kinetics regarding H^+ pumping activity warrants further study. To our knowledge, this is the first report showing that bLf inhibits the hydrolytic activity of V-ATPase.

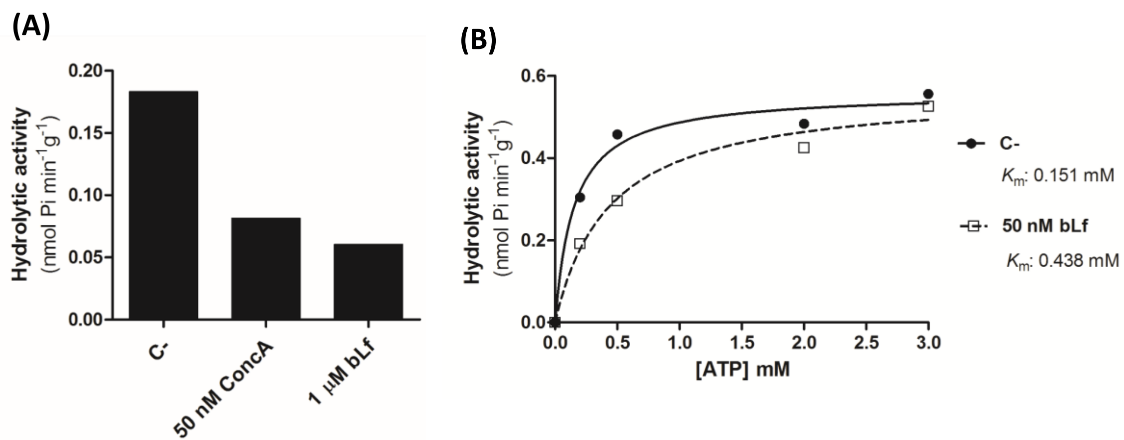


Figure II.8: Measurement of V-ATPase hydrolytic activity upon exposure to bLf and ConcA in lysosomes purified from rat liver. (A) Hydrolytic activity was measured in control lysosomes and incubated with 50 nM ConcA or 1μM bLf for 30 min at 37 °C. **(B)** Hydrolytic activity kinetics in the presence and absence of 50 nM bLf. Lines are derived by fitting the data points to a Michaelis–Menten kinetics. These results were obtained in collaboration with M. Gonçalves and L. Loureiro.

Overall, these results demonstrate that bLf inhibits the V-ATPase H^+ pumping activity in a time- and concentration-dependent manner and also its hydrolytic activity. Therefore, bLf seems to act as a PPI by targeting the V-ATPase present at the plasma membrane of some metastatic cancer cells.

II.3. DISCUSSION

Lf can be obtained from different sources such as human, bovine, camel or sheep (El-Fakharany et al., 2013). Though in the current work we have used Lf from bovine origin and poorly saturated with iron (21%), the results obtained will be compared with studies in which Lf from human origin was used since several evidences showed that bLf and hLf have similar biological effects in different circumstances (Buccigrossi et al. 2007; Liao et al. 2012; El-Fakharany et al. 2013). The use of bLf over hLf provides many advantages namely in what concerns its availability, commercial potential and administration. In 2009, Lf production from bovine milk, by manufacturing companies, was assumed to be more than 60 t/yr (Tomita et al., 2009). Remarkably, the European Food Safety Authority has already approved bLf as a safe ingredient for various alimentary products (EFSA-NDA, 2012). In fact, bLf is nowadays added as a component of many products like yogurts, dietary supplements, cosmetic products, milk-type drinks, infant formulae, pet food, among others (Tomita et al., 2009). Some of these products are considered nutraceuticals which, in addition to nutritional roles, can exert biological functions to promote health and prevent disease, including cancer (de Mejia and Dia, 2010). Oral administration of bLf has been extensively studied and many reports demonstrated its preventive and therapeutic role (reviewed by Tomita et al. 2009).

The bLf mediated inhibition of cellular proliferation and induction of apoptosis of cancer cell lines is in line with previous studies. Actually, Lf has been shown to have anti-tumoral activity in many types of cancer (see chapter I), but in the present study we focused on breast cancer, one of the leading causes of death worldwide (WHO, 2014). Moreover, we addressed the bLf cytotoxicity to non-tumorigenic cells to assess its specificity to cancer cells. Given the existence of previous reports, including on two of the cell lines used throughout this study, describing bLf-induced apoptosis associated with different markers, herein we only evaluated the protein effect on proliferation and in the appearance of a sub-G0/G1 peak to ensure that apoptosis was triggered under our experimental conditions. Indeed, our group have previously shown that other doses of bLf (0.125-125 µM) can induce apoptosis in Hs 578T and T-47D cell lines by a 2-fold increase in caspase-3 and caspase-7 activity (Duarte et al., 2011). We also recently reported that bLf can inhibit the viability and induce apoptosis of MCF-7 breast cancer

cells as characterized by phosphatidylserine externalisation, depolarisation of mitochondrial membrane potential and decreased expression of the anti-apoptotic protein Bcl-2 (Zhang et al., 2014a). In addition, Damiens and collaborators showed that hLf inhibits proliferation and induces cell cycle arrest at G1 to S transition and affects the levels of various cell cycle regulatory proteins in breast carcinoma MDA-MB-231 cells (Damiens et al., 1999). On the other hand, a recombinant adenovirus expressing hLf cDNA was found to induce a G1 cell cycle arrest in MCF-7 breast cancer cells, the appearance of a sub-G0/G1 peak indicative of apoptosis, phosphatidylserine externalisation, decrease in Bcl-2 protein levels and increase in the levels of pro-apoptotic Bax protein (Wang et al., 2012b). A similar hLf construct was applied in an *in vivo* study with mice harbouring EMT6 breast cancer cells, in which the protein administration was found to decrease the tumor growth and induce apoptosis that is also characterized by the appearance of a sub-G0/G1 peak, alteration of Bcl-2, Bax and caspase-3 expression (Wang et al., 2011). In the present work, although for the bLf-treated Hs 578T cells there seems to be a greater accumulation of cells in the S phase, the differences in the cells distribution among the cell cycle phases were not significant. However, the appearance of an apoptotic sub-G0/G1 peak was evident in the cancer cell lines in a time- and concentration-dependent manner. Considering the studies reported in the literature and our own results, it is reasonable to infer that, under the working conditions used in this study, bLf induces apoptosis and probably alters the expression levels of some apoptosis-related proteins. It is also noteworthy that MDA-MB-231 and EMT6 breast cancer cell lines display a highly metastatic ability, which is in line with the higher susceptibility of the invasive cell line Hs 578T verified in our conditions. Recently, a large scale proteomic approach was carried out on MDA-MB-231 breast carcinoma cells treated with hLf. The classification of the upregulated proteins in hLf-treated cells showed that a great part of the total protein pool was involved in the maintenance of cellular homeostasis with proteins involved in cell signalling, cell cycle and apoptosis, which is in total agreement with all the aforementioned results, including our own (Hoedt et al., 2014). Even though several researchers have studied the anti-tumoral activity of Lf, the molecular mechanism underlying such activity is still elusive.

Regarding the susceptibility of the three breast cell lines to bLf, it seems to be related with their differential invasion ability, as the invasive cells were more sensitive

than the non-invasive, and the non-tumorigenic cells were found to be resistant. Indeed, our results suggest that specific features of highly metastatic cells namely the V-ATPase cellular distribution at the plasma membrane and the extracellular acidification capacity (Sennoune et al., 2004) underlie these susceptibilities. In fact, the most sensitive cell line to bLf, Hs 578T, being highly invasive, exhibits a higher acidification capacity than the other cells, which is perfectly corroborated by a previous study that demonstrates the same trend using other breast cell lines (Montcourrier et al., 1997). Moreover, we confirmed that these cells are the only ones displaying V-ATPase at the plasma membrane. The first reference to the V-ATPase re-localization in cancer cells dates back to the early 90s (Martinez-Zaguilan et al., 1993). Since then, more data have been reported that supports this evidence, specifically on the breast cancer invasive MDA-MB-231 and MCF10CA1a cells, for which it was proved that the V-ATPase is much more prominent at the plasma membrane of the metastatic cells than in the poorly invasive cells MCF-7 and MCF10a, like it occurs in the cell lines under study (Sennoune et al., 2004; Capecci and Forgac, 2013). V-ATPase subunits *a4* and *a3*, respectively, were the ones identified as responsible for such a plasma membrane translocation in these two highly metastatic cell lines (Hinton et al., 2009; Capecci and Forgac, 2013). Furthermore, in MDA-MB-231 cell line it was also shown that subunit C1 of V-ATPase facilitates cancer metastasis by manipulation of F-actin cytoskeleton arrangement (Feng et al., 2014). Accordingly, it is now well accepted that the unique localization of V-ATPase at the plasma membrane in tumor cells directly contributes to the metastatic phenotype and constitutes a mechanism for homeostatic adaptation to the adverse TME (Sennoune et al., 2004; De Milito et al., 2010).

In accordance with the suggested V-ATPase involvement in bLf-induced apoptosis of Hs 578T cells, we found that bLf, like ConcA, induces extracellular alkalinisation in these cells. This effect on pHe is associated with the intracellular acidification induced by bLf in the same cells. A cytosolic acidification triggered by Lf was previously observed in *C. albicans* cells exposed to hLf (Viejo-Díaz et al., 2004). However, this is the first time that bLf is described to increase the extracellular pH of cancer cells. In view of this evidence, we speculated that bLf acts as a PPI, small drugs that specifically target H^+-ATPases inhibiting its mediated proton translocation (Fais et al., 2007). It was demonstrated that the PPI esomeoprazol leads to intracellular acidification in human

melanoma cells both *in vitro* and *in vivo*, significantly impacting the TME and the TME-dependent tumor invasion (De Milito et al., 2010). Since bLf exhibited similar effects on pH, it is likely to have identical outcomes that could be associated with a V-ATPase inhibition.

V-ATPase was found to be very abundant in the lipid rafts of highly metastatic melanoma cells (Baruthio et al., 2008). Therefore, we surveyed the effect of bLf in lipid rafts distribution in the three cell lines under study, and found that bLf, like the cholesterol-depleting agent MβCD, alters cholesterol-enriched lipid rafts distribution. In fact, when Hs 578T cells were incubated with bLf, the membrane staining greatly decreased whereas an intracellular punctate pattern appeared, thus indicating internalization of cholesterol and possibly of lipid rafts. A similar effect was observed with the anti-tumor agent edelfosine as treatment with this compound caused internalization and re-distribution of lipid rafts in HeLa cells (Mollinedo et al., 2011).

The only study reporting the Lf possible interaction with lipid rafts regards the brush border of the small intestine, a specialized membrane that owns a high content of glycolipid-based lipid rafts. These structures are stabilized by intelectin, a trimeric lectin that also functions as an intestinal Lf receptor (Danielsen and Hansen, 2008). Lf, by interacting with this receptor may interfere with lipid raft stability. Our hypothesis for such an evident effect of bLf in lipid rafts of the metastatic Hs 578T cells is that, like it happens on melanoma cells (Baruthio et al., 2008), the V-ATPase is localized in the lipid rafts of these cells. By inhibiting V-ATPase, bLf alters the stability of lipid rafts leading to their internalization. However, the possibility of a direct effect of bLf on lipid rafts cannot be excluded. For example, bLf can have a similar effect to MβCD, which was shown to inhibit V-ATPase activity by depleting cholesterol in both osteoclast and synaptic vesicles (Yoshinaka et al., 2004; Ryu et al., 2010), and not through a direct inhibition of V-ATPase.

These former results encouraged us to further investigate whether V-ATPase is a bLf target. To this end, we studied the bLf effect on V-ATPase H^+ pumping activity in crude membrane fractions prepared from T-47D cell line and in rat liver lysosomes suspensions. In both cases we found that the electrochemical gradient generated after addition of ATP was dissipated by bLf. In the crude membrane fraction, the inhibitory effect of bLf was greater than the two V-ATPase inhibitors, ConcA and BafA1, which

probably indicates that, besides V-ATPase, other ion pumps are affected by bLf at the concentration used. This is likely a consequence of the diversity of cellular membranes present in these fractions. However, we were able to demonstrate in a clean model of isolated lysosomes that, in fact, bLf inhibits V-ATPase H^+ pumping activity.

We also studied the consequences of bLf incubation on the hydrolytic activity in isolated lysosomes. Surprisingly, we observed that bLf inhibits V-ATPase hydrolytic activity to a similar level as ConcA. Despite the fact that the ConcA binding site to V-ATPase is in the *c* subunit of the proton translocating sector (V_0), ConcA and also BafA1 have been repeatedly reported to also inhibit hydrolytic activity in a similar concentration range to that used in our experiments (Bowman and Bowman, 2002; Huss et al., 2002; Bowman et al., 2004). Moreover, ConcA effect is often used as a control to determine the specific V-ATPase hydrolytic activity (Zhang et al., 2012).

Altogether, the latter results indicate that V-ATPase is a bLf target. Some reports in bacteria and fungi have already pointed to an interaction between Lf and H^+ pumps. Actually, hLf was previously reported to inhibit proton pumping and ATPase activity of the F-type H^+-ATPase in inverted membrane vesicles and plasma membrane fractions from *L. lactis*, respectively (Andrés and Fierro, 2010). The authors proposed that bLf targets this pump, which underlies its *in vitro* bactericidal activity. Also, in the fungus *P. expansum*, an interaction between Lf and P-type H^+-ATPases was suggested (Wang et al., 2012a).

Given all the interesting data that could be obtained, we suggest that the *in vitro* approach set up with isolated lysosomes constitute an excellent tool to mimic the metastatic cancer cells that display V-ATPase at the plasma membrane, as well as to study and extrapolate the effect of drugs in the V-ATPase activity. Obviously, in cancer cells the topological orientation of this pump localized at the plasma membrane is inverted when compared to lysosomes, but this difference can be overcome by the study of isolated plasma membranes inverted vesicles.

As a whole, we demonstrated that the molecular mechanism underlying the widely studied Lf anti-tumoral activity relies on its targeting and inhibition of both V-ATPase H^+ pumping and hydrolytic activity. By targeting V-ATPase, bLf induces intracellular acidification and extracellular alkalinisation due to cytosolic H^+ accumulation (see Fig. II.9 for our working model). After this first event many cascades

mediated by intracellular acidification may be triggered. For instance, acidic intracellular pH was found to induce re-distribution and release of active cathepsin B from lysosomes in murine and human cancer cells (Rozhin et al., 1994). On the other hand, acidic pHi-induced cell death was demonstrated to strongly induce Bax translocation to the mitochondria (Yang et al., 2008). Perturbation of pHi affects many cellular functions such as the control of DNA synthesis, cellular proliferation, protein synthesis rate and apoptosis (Nakamura et al., 2006).

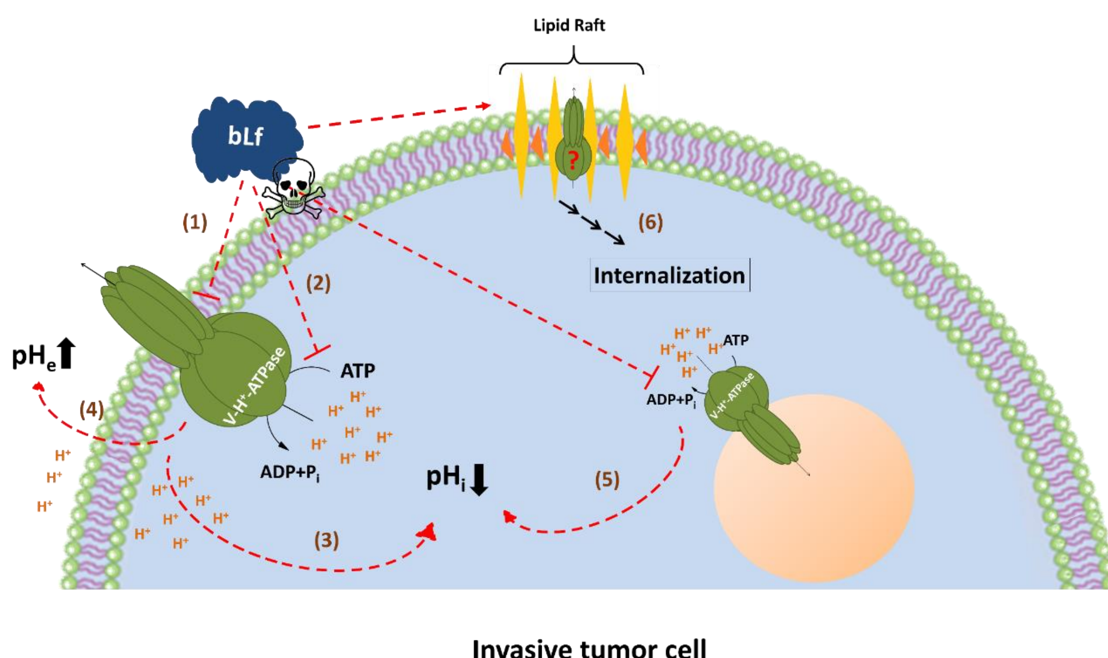


Figure II.9: Working model on the molecular mechanism underlying the anti-tumoral activity of Lf. The exposure of invasive cancer cells to bLf leads to the inhibition of plasma membrane V-ATPase proton pumping (1) and hydrolytic (2) activities. Consequently, V-ATPase is no longer capable to translocate H^+ to the extracelular space, which leads to intracelular acidification (3) and extracelular alkalinisation (4). The hypothetical lysosomal V-ATPase inhibition by Lf or its derived peptides can also contribute to the decresase of pHi (5). Moreover, Lf can disrupt lipid rafts leading to its internalization (6), which can or cannot be a consequence of the V-ATPase inhibition.

By unveiling a molecular mechanism underlying bLf anti-tumoral activity, our data uncovered a novel Lf activity, PPI-like activity. The most relevant feature of Lf that perfectly suits our model and this PPI-like activity is that it has been reported to be more active at acidic pH. As a matter of fact, hLf anti-bacterial and anti-fungal activities were found to be more effective at pH 5.5 than at pH 7.4 (Viejo-Díaz et al., 2004; Andrés and

Fierro, 2010). Hence, bLf might have a preferential effect for tissues characterised by low pHe like tumors and its surrounding acidic microenvironment. On the other hand, another aspect that may strengthen our proposal that plasma membrane V-ATPase is a bLf target, is the fact that this protein does not internalize into some cancer cells. Although this is a controversial subject, more evidence that supports this fact has been published. Exogenously added bLf is not internalized by MCF-7 breast cancer cells (Baumrucker et al., 2006), and our group's previous results in T-47D and MDA-MB-231 breast cancer cells also indicate that bLf mainly remains outside the cell, with only a small percentage being internalized and accumulated in vesicle-like structures (Zhang et al., 2014b). Lf PPI-like activity can also be the key to explain the anti-metastatic role of Lf (Bezault et al., 1994; Iigo et al., 1999; Wolf et al., 2003; Tung et al., 2013) and may have particular interest on therapeutic strategies against highly metastatic tumors.

Another key outcome from our work concerns the bLf specific targeting of cancer cells because, at least in our experimental conditions, it exhibited no effect against a non-tumorigenic epithelial breast cell line. Interestingly, this aspect is corroborated with some clinical trials already performed that attest the safety and well-tolerance of Lf treatment with no serious adverse effects being identified (Varadhachary et al., 2004; Kozu et al., 2009; Macciò et al., 2010; Moastafa et al., 2014). Can the concept of "magic bullet" described all those years ago by Paul Ehrlich (Ehrlich, 1906), be applied to Lf, this naturally occurring protein?

In summary, our data show that the three studied breast cell lines display distinct susceptibilities to bLf with the non-tumorigenic and the invasive cell line displaying the lowest and the highest sensitivity, respectively. Since we found that V-ATPase localizes at the plasma membrane of the invasive cells, we propose that the different susceptibilities of cancer and normal cells to bLf stems from the differential cellular localization of this pump and its inhibition by bLf. In fact, we demonstrate for the first time that bLf, similarly to the classic V-ATPase inhibitors ConcA and BafA1, inhibits this pump in crude membranes isolated from a breast cancer cell line and in lysosomes purified from rat liver. Therefore, we propose that bLf displays PPI-like activity by targeting and inhibiting the V-ATPase present at the cell surface of the invasive tumor cells, possibly localized on lipid rafts, and that this activity underlies the specificity of bLf to cancer cells and may have an important impact on the TME.

II.4. REFERENCES

Andrés, M.T., and J.F. Fierro. 2010. Antimicrobial mechanism of action of transferrins: selective inhibition of H^+-ATPase. *Antimicrob. Agents Chemother.* 54:4335–42. doi:10.1128/AAC.01620-09.

Arai, K., A. Shimayasg, N. Hiratanill, and S. Ohkumaji. 1993. Purification and Characterization of Lysosomal H^+-ATPase. *J. Biol. Chem.* 268:5649–5660.

Baruthio, F., M. Quadroni, C. Rüegg, and A. Mariotti. 2008. Proteomic analysis of membrane rafts of melanoma cells identifies protein patterns characteristic of the tumor progression stage. *Proteomics*. 8:4733–47. doi:10.1002/pmic.200800169.

Baumrucker, C.R., F. Schanbacher, Y. Shang, and M.H. Green. 2006. Lactoferrin interaction with retinoid signaling: cell growth and apoptosis in mammary cells. *Domest. Anim. Endocrinol.* 30:289–303. doi:10.1016/j.domaniend.2005.07.009.

Bezault, J., R. Bhimani, and J. Wiprovnick. 1994. Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice. *Cancer Res*. 2310–2312.

Bowman, B.J., and E.J. Bowman. 2002. Mutations in subunit C of the vacuolar ATPase confer resistance to bafilomycin and identify a conserved antibiotic binding site. *J. Biol. Chem.* 277:3965–72. doi:10.1074/jbc.M109756200.

Bowman, E.J., L. a Graham, T.H. Stevens, and B.J. Bowman. 2004. The bafilomycin/concanamycin binding site in subunit *c* of the V-ATPases from *Neurospora crassa* and *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279:33131–8. doi:10.1074/jbc.M404638200.

Buccigrossi, V., G. de Marco, E. Bruzzese, L. Ombrato, I. Bracale, G. Polito, and A. Guarino. 2007. Lactoferrin induces concentration-dependent functional modulation of intestinal proliferation and differentiation. *Pediatr. Res.* 61:410–4. doi:10.1203/pdr.0b013e3180332c8d.

Capecci, J., and M. Forgac. 2013. The function of vacuolar ATPase (V-ATPase) *a* subunit isoforms in invasiveness of MCF10a and MCF10CA1a human breast cancer cells. *J. Biol. Chem.* 288:32731–41. doi:10.1074/jbc.M113.503771.

Damiens, E., I. El Yazidi, J. Mazurier, I. Duthille, G. Spik, and Y. Boilly-Marer. 1999. Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. *J. Cell. Biochem.* 74:486–98.

Danielsen, E.M., and G.H. Hansen. 2008. Lipid raft organization and function in the small intestinal brush border. *J. Physiol. Biochem.* 64:377–382. doi:10.1007/BF03174093.

de Mejia, E.G., and V.P. Dia. 2010. The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Rev.* 29:511–28. doi:10.1007/s10555-010-9241-4.

De Milito, A., R. Canese, M.L. Marino, M. Borghi, M. Iero, A. Villa, G. Venturi, F. Lozupone, E. Iessi, M. Logozzi, P. Della Mina, M. Santinami, M. Rodolfo, F. Podo, L. Rivoltini, and S. Fais. 2010. pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor acidity. *Int. J. Cancer*. 127:207–19. doi:10.1002/ijc.25009.

Duarte, D.C., a Nicolau, J. a Teixeira, and L.R. Rodrigues. 2011. The effect of bovine milk lactoferrin on human breast cancer cell lines. *J. Dairy Sci.* 94:66–76. doi:10.3168/jds.2010-3629.

EFSA-NDA, European Food Safety Authority - Dietetic Products, Nutrition and Allergies 2012. Scientific opinon on bovine lactoferrin. *EFSA J.* 10.

Ehrlich P. 1906. The relations existing between chemical constitution, distribution and pharmacological action. *Collected studies on Immunity*. 404-43.

El-Fakharany, E.M., L. Sánchez, H. a Al-Mehdar, and E.M. Redwan. 2013. Effectiveness of human, camel, bovine and sheep lactoferrin on the hepatitis C virus cellular infectivity: comparison study. *Virol. J.* 10:199. doi:10.1186/1743-422X-10-199.

Fais, S., A. De Milito, H. You, and W. Qin. 2007. Targeting vacuolar H^+-ATPases as a new strategy against cancer. *Cancer Res.* 67:10627–30. doi:10.1158/0008-5472.CAN-07-1805.

Feng, S., M. Cai, P. Liu, L. Wei, J. Wang, J. Qi, and L. Deng. 2014. Atp6v1c1 may regulate filament actin arrangement in breast cancer cells. *PLoS One*. 9:e84833. doi:10.1371/journal.pone.0084833.

Hinton, A., S.R. Sennoune, S. Bond, M. Fang, M. Reuveni, G.G. Sahagian, D. Jay, R. Martinez-Zaguilan, and M. Forgac. 2009. Function of a subunit isoforms of the V-ATPase in pH homeostasis and in vitro invasion of MDA-MB231 human breast cancer cells. *J. Biol. Chem.* 284:16400–8. doi:10.1074/jbc.M901201200.

Hoedt, E., K. Chaoui, I. Huvent, C. Mariller, B. Monsarrat, O. Burlet-schiltz, and A. Pierce. 2014. SILAC-Based Proteomic Profiling of the Human MDA-MB- 231 Metastatic Breast Cancer Cell Line in Response to the Two Antitumoral Lactoferrin Isoforms : The Secreted Lactoferrin and the Intracellular Delta-Lactoferrin. *PLoS One*. 9(8): e104563. doi:10.1371/journal.pone.0104563.

Huss, M., G. Ingenhorst, S. König, M. Gassel, S. Dröse, A. Zeeck, K. Altendorf, and H. Wieczorek. 2002. Concanamycin A, the specific inhibitor of V-ATPases, binds to the V(o) subunit c. *J. Biol. Chem.* 277:40544–8. doi:10.1074/jbc.M207345200.

Iigo, M., T. Kuhara, Y. Ushida, K. Sekine, and M.A. Moore. 1999. Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. *Clin Exp Metastasis.* 17(1):35-40.

Jefferies, K.C., D.J. Cipriano, and M. Forgac. 2009. Function, structure and regulation of the vacuolar (H+)-ATPases. *Arch Biochem Biophys.* 476:33–42. doi:10.1016/j.abb.2008.03.025.Function.

Kozu, T., G. Iinuma, Y. Ohashi, Y. Saito, T. Akasu, D. Saito, D.B. Alexander, M. Iigo, T. Kakizoe, and H. Tsuda. 2009. Effect of orally administered bovine lactoferrin on the growth of adenomatous colorectal polyps in a randomized, placebo-controlled clinical trial. *Cancer Prev. Res. (Phila).* 2:975–83. doi:10.1158/1940-6207.CAPR-08-0208.

Lee, S., S. Won, C. Pyo, N. Yoo, J. Kim, and S. Choi. 2009. Requirement of the JNK-associated Bcl-2 pathway for human lactoferrin-induced apoptosis in the Jurkat leukemia T cell line. *Biochimie*. 91:102–108. doi:10.1016/j.biochi.2008.05.004.

Li, Y.C., M.J. Park, S.K. Ye, C.W. Kim, and Y.N. Kim. 2006. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. *Am. J. Pathol.* 168:1107–18; quiz 1404–5. doi:10.2353/ajpath.2006.050959.

Liao, Y., R. Jiang, and B. Lönnerdal. 2012. Biochemical and molecular impacts of lactoferrin on small intestinal growth and development during early life. *Biochem. Cell Biol.* 90:476–84. doi:10.1139/o11-075.

Lowry, O.H., N.J. Rosebrough, L. Farr, and R.J. Randall. 1951. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* 193.

Macciò, A., C. Madeddu, G. Gramignano, C. Mulas, E. Sanna, and G. Mantovani. 2010. Efficacy and safety of oral lactoferrin supplementation in combination with rHuEPO-beta for the treatment of anemia in advanced cancer patients undergoing chemotherapy: open-label, randomized controlled study. *Oncologist*. 15:894–902. doi:10.1634/theoncologist.2010-0020.

Martinez-Zaguilan, R., R.M. Lynch, G.M. Martinez, and R.J. Gillies. 1993. Vacuolar-type H(+)-ATPases are functionally expressed in plasma membranes of human tumor cells. *Am J Physiol Cell Physiol*. 265:C1015–1029.

Moastafa, T.M., A.E. El-Sissy, G.K. El-Saeed, and M.S. Koura. 2014. Study on the Therapeutic Benefit on Lactoferrin in Patients with Colorectal Cancer Receiving Chemotherapy. *Int. Sch. Res. Not.* 2014:1–10. doi:10.1155/2014/184278.

Mollinedo, F., M. Fernández, V. Hornillos, J. Delgado, F. Amat-Guerri, a U. Acuña, T. Nieto-Miguel, J. a Villa-Pulgarín, C. González-García, V. Ceña, and C. Gajate. 2011. Involvement of lipid rafts in the localization and dysfunction effect of the antitumor

ether phospholipid edelfosine in mitochondria. *Cell Death Dis.* 2:e158. doi:10.1038/cddis.2011.41.

Montcourrier, P., I. Silver, R. Farnoud, I. Bird, and H. Rochefort. 1997. Breast cancer cells have a high capacity to acidify extracellular milieu by a dual mechanism. *Clin. Exp. Metastasis*. 15:382–92.

Nakamura, U., M. Iwase, Y. Uchizono, K. Sonoki, N. Sasaki, H. Imoto, D. Goto, and M. Iida. 2006. Rapid intracellular acidification and cell death by H_2O_2 and alloxan in pancreatic beta cells. *Free Radic. Biol. Med.* 40:2047–55. doi:10.1016/j.freeradbiomed.2006.01.038.

Neri, D., and C.T. Supuran. 2011. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat. Rev. Drug Discov.* 10:767–77. doi:10.1038/nrd3554.

Paffett, M.L., J.S. Naik, M. a Riddle, S.D. Menicucci, A.J. Gonzales, T.C. Resta, and B.R. Walker. 2011. Altered membrane lipid domains limit pulmonary endothelial calcium entry following chronic hypoxia. *Am. J. Physiol. Heart Circ. Physiol.* 301:H1331–40. doi:10.1152/ajpheart.00980.2010.

Rozhin, J., M. Sameni, G. Ziegler, and B.F. Sloane. 1994. Pericellular pH Affects Distribution and Secretion of Cathepsin B in Malignant Cells Pericellular pH Affects Distribution and Secretion of Cathepsin B in Malignant Cells. *Cancer Res*. 6517–6525.

Ryu, J., H. Kim, E.-J. Chang, H.J. Kim, Y. Lee, and H.-H. Kim. 2010. Proteomic analysis of osteoclast lipid rafts: the role of the integrity of lipid rafts on V-ATPase activity in osteoclasts. *J. Bone Miner. Metab.* 28:410–7. doi:10.1007/s00774-009-0150-y.

Sennoune, S.R., K. Bakunts, G.M. Martínez, J.L. Chua-Tuan, Y. Kebir, M.N. Attaya, and R. Martínez-Zaguilán. 2004. Vacuolar H^+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. *Am. J. Physiol. Cell Physiol.* 286:C1443–52. doi:10.1152/ajpcell.00407.2003.

Tomita, M., H. Wakabayashi, K. Shin, K. Yamauchi, T. Yaeshima, and K. Iwatsuki. 2009. Twenty-five years of research on bovine lactoferrin applications. *Biochimie*. 91:52–7. doi:10.1016/j.biochi.2008.05.021.

Troost F, Steijns J, Saris W, Brummer, R.M. 2001. Gastric Digestion of Bovine Lactoferrin In Vivo in Adults. *J. Nutr.* 1:2101–2104.

Tsuda, H., T. Kozu, G. Iinuma, and Y. Ohashi. 2010. Cancer prevention by bovine lactoferrin : from animal studies to human trial. 399–409. doi:10.1007/s10534-010-9331-3.

Tung, Y.-T., H.-L. Chen, C.-C. Yen, P.-Y. Lee, H.-C. Tsai, M.-F. Lin, and C.-M. Chen. 2013. Bovine lactoferrin inhibits lung cancer growth through suppression of both

inflammation and expression of vascular endothelial growth factor. *J. Dairy Sci.* 96:2095–106. doi:10.3168/jds.2012-6153.

Varadhachary, A., J.S. Wolf, K. Petrak, B.W. O'Malley, M. Spadaro, C. Curcio, G. Forni, and F. Pericle. 2004. Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. *Int. J. Cancer*. 111:398–403. doi:10.1002/ijc.20271.

Vera-estrella, R., B.J. Barkla, J. Higgins, and E. Blumwald. 1994. Plant Defense Response to Fungal Pathogens. *Plant Physiol.* 104:209–215.

Viejo-Díaz, M., M.T. Andrés, and J.F. Fierro. 2004. Modulation of in vitro fungicidal activity of human lactoferrin against *Candida albicans* by extracellular cation concentration and target cell metabolic activity. *Antimicrob. Agents Chemother.* 48:1242–8. doi:10.1128/AAC.48.4.1242.

Wang, J., Q. Li, Y. Ou, Z. Han, K. Li, P. Wang, and S. Zhou. 2011. Inhibition of tumor growth by recombinant adenovirus containing human lactoferrin through inducing tumor cell apoptosis in mice bearing EMT6 breast cancer. *Arch. Pharm. Res.* 34:987–95. doi:10.1007/s12272-011-0616-z.

Wang, J., X. Shi, H. Wang, X. Xia, and K. Wang. 2012a. Effects of Esterified Lactoferrin and Lactoferrin on Control of Postharvest Blue Mold of Apple Fruit and Their Possible Mechanisms of Action. *J. Agric. Food Chem.* 60:6432–6438.

Wang, J., Q. Li, Y. Ou, K. Li, Z. Han, P. Wang, and S. Zhou. 2012b. Recombination adenovirus-mediated human lactoferrin cDNA inhibits the growth of human MCF-7 breast cancer cells. *J. Pharm. Pharmacol.* 64:457–63. doi:10.1111/j.2042-7158.2011.01410.x.

WHO, 2014 – World Health Organization: http://www.who.int/mediacentre/factsheets/fs297/en/ (visited on 13 November 2014)

Wolf, J.S., D. Li, R.J. Taylor, and B.W. O'Malley, Jr. 2003. Lactoferrin Inhibits Growth of Malignant Tumors of the Head and Neck. *Orl J Otorhinolaryngol Relat Spec.* 65:245–249. doi:10.1159/000075220.

Xiao, Y., C.L. Monitto, K.M. Minhas, Y. Xiao, C.L. Monitto, K.M. Minhas, and D. Sidransky. 2004. Lactoferrin Down-Regulates G 1 Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells Lactoferrin Down-Regulates G 1 Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells. *Clinic Cancer Res.* 10:8683–8686.

Yang, L., Y. Mei, Q. Xie, X. Han, F. Zhang, L. Gu, Y. Zhang, Y. Chen, G. Li, and Z. Gao. 2008. Acidification induces Bax translocation to the mitochondria and promotes ultraviolet light-induced apoptosis. *Cell. Mol. Biol. Lett.* 13:119–29. doi:10.2478/s11658-007-0042-x.

Yoshinaka, K., H. Kumanogoh, S. Nakamura, and S. Maekawa. 2004. Identification of V-ATPase as a major component in the raft fraction prepared from the synaptic plasma membrane and the synaptic vesicle of rat brain. *Neurosci. Lett.* 363:168–72. doi:10.1016/j.neulet.2004.04.002.

Zhang, M., Y. Fang, Z. Liang, and L. Huang. 2012. Enhanced expression of vacuolar H^+-ATPase subunit E in the roots is associated with the adaptation of *Broussonetia papyrifera* to salt stress. *PLoS One*. 7:e48183. doi:10.1371/journal.pone.0048183.

Zhang, Y., C.F. Lima, and L.R. Rodrigues. 2014a. In vitro evaluation of bovine lactoferrin potential as an anticancer agent. *Int. Dairy J.* 40:6–15. doi:10.1016/j.idairyj.2014.08.016.

Zhang, Y., A. Nicolau, C.F. Lima, and L.R. Rodrigues. 2014b. Bovine Lactoferrin Induces Cell Cycle Arrest and Inhibits Mtor Signaling in Breast Cancer Cells. *Nutr. Cancer*. 0:1–15. doi:10.1080/01635581.2014.956260.

CHAPTER III

Final Remarks and Future Perspectives

III.1. FINAL REMARKS

In this thesis project, we aimed to dissect the molecular mechanism underlying the widely studied Lf anti-tumoral activity. Various mechanisms of action were already proposed such as induction of apoptosis, cell cycle arrest, modulation of the immune system, among others. However, the cancer cells molecular target with which Lf interacts to exert its anti-tumoral effect in such a great number of human tumors, has not yet been identified. Besides its anti-tumoral activity, many other functions have been attributed to this protein. Some studies on the effects of bLF on bacteria and fungi particularly caught our attention. In these studies, an inhibitory interaction between Lf and H^+-ATPases was suggested to mediate the protein anti-microbial and anti-fungal activities. Since these proton pumps share a high similarity with the eukaryotic proton pump V-ATPase, we wondered whether in cancer cells, such an interaction could also exist. All the work done throughout this study was thought to clarify and possibly confirm this hypothesis.

Given the well-known importance of V-ATPase in breast cancer progression and lack of mechanistic studies concerning Lf and breast cancer, we used in this work two breast cancer cell lines with distinct metastatic ability, and one non-tumorigenic to be used as a control of normal epithelial breast cells. Our first results, concerning the susceptibilities of these cells to bLf, encouraged us to proceed with the investigation of our hypothesis as the highly metastatic cell line was much more susceptible to bLf than the other two. Since in breast cancer, highly metastatic cells were found to display an overexpressed plasma membrane V-ATPase, we next assessed this in the cell lines under study, and confirmed that the most sensitive cell line was the only one with a plasma membrane localization of this proton pump. With these results, a relation between V-ATPase and susceptibility to bLf seemed to make perfect sense. Importantly, bLf exhibited no cytotoxic effect against the non-tumorigenic cells, being therefore specific for the cancer cells.

Since V-ATPase plays a fundamental role in maintaining pH homeostasis, the next logical step was to monitor both pHe and pHi in bLf-treated cells. What we observed was in total agreement with our hypothesis, i.e., upon bLf exposure, an extracellular alkalinisation and an intracellular acidification occurs in accordance with the inhibition

of V-ATPase by bLf. Other evidence that enthused us was that bLf altered the distribution of lipid rafts only in the invasive cell line, which is in line with the reported localization of V-ATPase in the lipid rafts fraction of highly metastatic cells. Following these observations, a direct evidence of the inhibition of this proton pump by bLf was missing. However, we were able to demonstrate in isolated crude membranes and lysosomes that bLf, in fact, inhibits both proton pumping and hydrolytic activity of V-ATPase.

As a whole, our data provided the first proof that bLf targets and inhibits V-ATPase, which we suggest might underlie the anti-tumoral activity of Lf mainly in invasive cancer cells displaying V-ATPase at the plasma membrane. In non-invasive cancer cells where this localization of V-ATPase is not observed, bLf inhibition can potentially be caused by a small amount of internalized Lf, or even through the entrance of Lf-digested peptides with similar activity.

Finally, we propose that the unique localization of V-ATPase at the plasma membrane, associated with the presumed higher activity of bLf in the acidic TME of cancer cells, may contribute to its PPI-like activity and explain its specificity against invasive cancer cells. By diminishing TME acidity, Lf could not only contribute to the inhibition of tumor growth and progression, but also to overcome the multidrug resistance, which constitutes a severe drawback of current cancer therapies.

III.2. FUTURE PERSPECTIVES

As future perspectives, much work is warranted in order to understand the implications of the newly discovered Lf PPI-like activity. Several research aspects should be addressed to better comprehend this Lf activity as discussed below. Firstly, it will be important to optimize the crude membrane isolation protocol in Hs 578T cell line to validate the V-ATPase inhibition in these cells. Also, the plasma membrane isolation followed by artificial vesicles formation of this cell line will allow to further support that bLf targets V-ATPase specifically localized at the plasma membrane. Experiments in medium with neutral (~7.4) and acidic (~5.0 or 6.0) extracellular pH will allow ascertaining if in cancer cells, like in microbial cells, Lf is also more active at acidic pH, which in turn will enhance specificity against cancer cells due to the acidic TME. The

assessment of other cellular events that may occur after Lf-induced V-ATPase inhibition and intracellular acidification, will unravel the intracellular cascades triggered by Lf. Preliminary results in our lab have shown that bLf perturbs plasma and mitochondrial membrane potential, but it would be interesting to monitor other cellular events such as lysosomal and mitochondrial membrane permeabilization and the release of cathepsins and mitochondrial pro-apoptotic factors to better characterize the cell death pathway triggered by bLf. Since all our experiments were carried with bLf containing low iron content, it would be interesting to investigate whether holo-bLf also displays PPI-like activity. In this way, it will be possible to ascertain if the inhibition of V-ATPase is affected by the Lf iron content and, possibly, to obtain insights into the inhibitory mechanism. The confirmation of bLf non-internalization in our cells is also important to understand the dynamics of bLf effect. If it is not internalized, the target(s) reside(s) at the cell surface and all the succeeding events occur as a consequence of that interaction. If cells can internalise bLf, even in a small amount, some long-term events may be triggered which could be a consequence of the interaction of bLf with other structures.

Further characterization of the novel bLf effect on lipid rafts is also worthwhile to understand the interplay between bLf, V-ATPase and lipid rafts, which could have crucial implications in cancer biology as cancer cells were found to have higher levels of cholesterol-rich lipid rafts than their normal counterparts. Investigating if V-ATPase is, in fact, at the lipid rafts in the invasive cell line Hs 578T will help to unveil whether the Lf-induced perturbation of lipid rafts is a consequence of V-ATPase inhibition or if rafts *per se* also constitute a direct Lf target. Other approaches can be used to better comprehend the role of lipid rafts in the anti-tumoral activity of bLf, namely lipid rafts disruption with methyl-β-ciclodextrin prior to bLf incubation, which will allow understanding if bLf cytotoxic effect depends on raft integrity, and determining the intracellular localization of lipid rafts after bLf incubation. As lipid rafts are important signalling platforms, exploring the intracellular cascades that are altered upon rafts perturbation by bLf, will constitute an innovative research topic that may uncover a new mechanism underlying the anti-tumoral activity of Lf.

Studies using various cell lines derived from other types of highly metastatic human cancers, and respective non-invasive and non-tumorigenic counterparts, would be very important to discover whether the Lf preferential cytotoxicity to metastatic cells

also occurs in other types of cancer or if it only occurs in breast cancer. In the sensitive metastatic cells, the determination of the V-ATPase localization will aid to realize if a similar mechanism to that we report here, also occurs in other cancer cells.

The deeper understanding of how the interaction between Lf and V-ATPase occurs is also crucial. Our first results suggest a direct interaction of bLf and V-ATPase based on the inhibition kinetics of the hydrolytic activity by bLf. This should also be ascertained for the V-ATPase H^+ pumping activity. The finding of which subunits and, ultimately, which residues are involved will also constitute an important scientific advance. Various approaches can be used for this purpose such as *in silico* protein-protein docking analysis to identify/restrict the number of candidate residues, and immunoprecipitation to confirm those residues involved in Lf-V-ATPase interaction, among others. In this regard, relevant clues may be provided by the use of yeast mutant strains harbouring V-ATPase point mutations in the most probable candidate residues.

Finally, it is imperative to obtain *in vivo* evidence of the effect of bLf on the V-ATPase and on the TME. For this purpose, mice bearing highly metastatic tumor cells that were previously demonstrated to be sensitive to Lf and to display V-ATPase at the plasma membrane may be used. ^1H magnetic resonance spectroscopy can be used to measure the extracellular pH of tumors in order to confirm if bLf, consistently with its induced V-ATPase inhibition, promotes the alkalinisation of the extracellular milieu. This *in vivo* mechanistic study is of great importance because drugs can have different actions and possibly diminished effects when in the body environment. Understanding if the targeting of Lf to cancer cells is sufficient to exert its anti-tumoral activity, or if a delivery system is required will also be interesting. Actually, some delivery systems based on nano- and microparticles are already being developed.

As Lf is a non-toxic and low-cost dietary protein, its application in healthcare and therapeutics is expected to be widespread. Thus, the research on its mechanisms of action is crucial for its safe and suitable application in humans. We believe that the findings obtained throughout this master thesis work will have great scientific impact and may be useful for future *in vitro* and *in vivo* studies on the anti-tumoral role of Lf.

CHAPTER IV

SUPPLEMENTAL MATERIAL

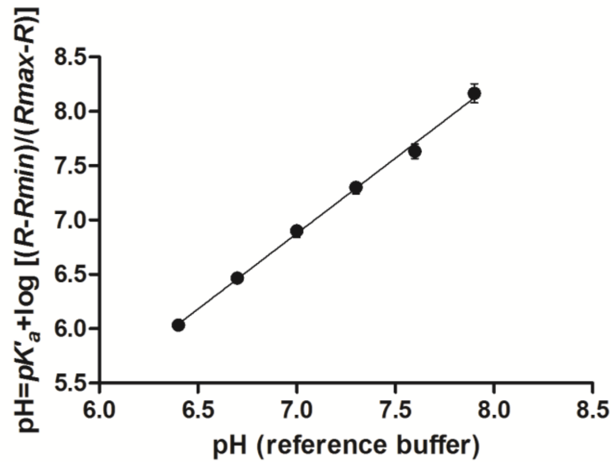


Figure IV.1: Calibration curve for the quantification of extracellular pH by Phenol Red absorbance at 562/450 nm. HBSS buffer containing 0.03 mM Phenol Red and 50 mM HEPES was imposed to a pH ranging from 6.4 to 8.2. pH values were calculated from the equation pH = pK'_a + log[(R – Rmin)/(Rmax – R)]. The calculated pH was plotted against the effective pH of HEPES-buffered reference solutions. Values correspond to mean ± S.E.M. of three independent experiments.

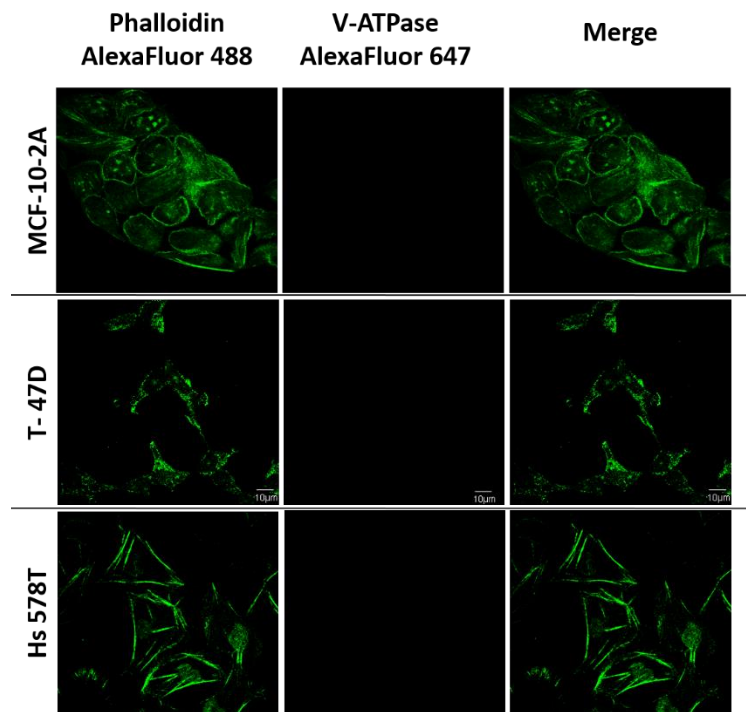


Figure IV.2: Panels correspond to the immunofluorescence negative controls in which the breast cells were incubated only with the secondary antibody Alexa fluor-647, thus demonstrating that in figure II.3 B the red labelling corresponds specifically to V-ATPase subunit *c'*. Cytoskeleton was labelled with Alexa fluor 488-Phalloidin (green fluorescence).

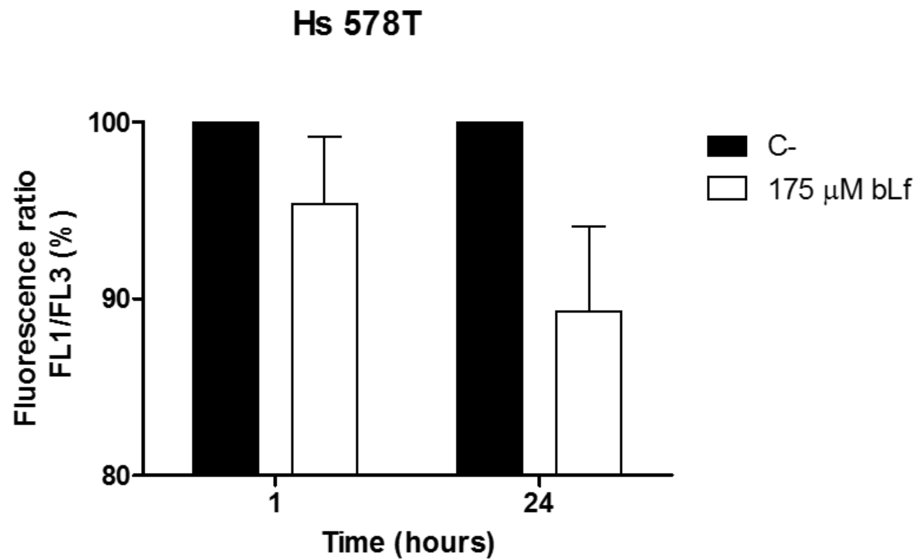


Figure IV.3: Effect of bLf (175 μM) treatment in the intracellular pH of the invasive breast cancer cell line Hs 578T along time. The intracellular pH was monitored by flow cytometry using the cytoplasmatic pH-sensitive dye BCECF-AM, as indicated by changes in the ratio between the mean green fluorescence intensity and the mean red fluorescence intensity (the lower the ratio the lower the pH). For each time point the ratio values were normalised to the respective untreated cells (negative control cells). bLf leads to intracellular acidification after 1h and 24h of incubation. Values are mean ± S.E.M. of two independent experiments and correspond to a preliminary result.

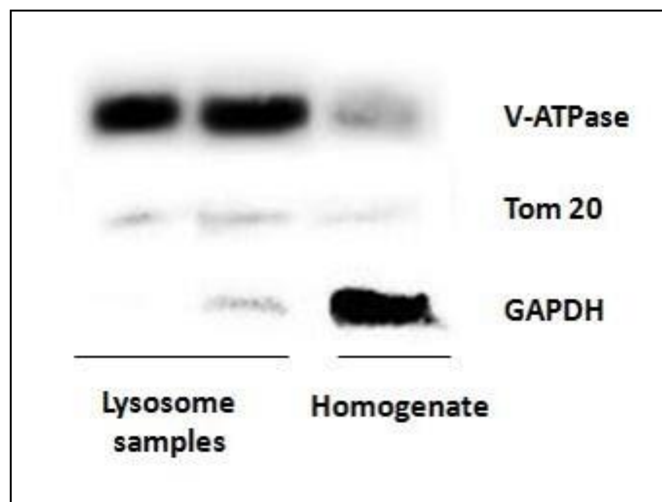


Figure IV.4: Purity of isolated rat liver lysosomes subcellular fraction monitored by western blot. Western blot analysis performed in a representative cell homogenate after mitochondria sedimentation and in two representative isolated lysosome fractions (50 μg protein/lane). Samples were labelled with antibodies specific for V-ATPase, used as a specific lysosomal marker; Tom20, a mitochondrial marker; and GAPDH, a cytosolic protein.

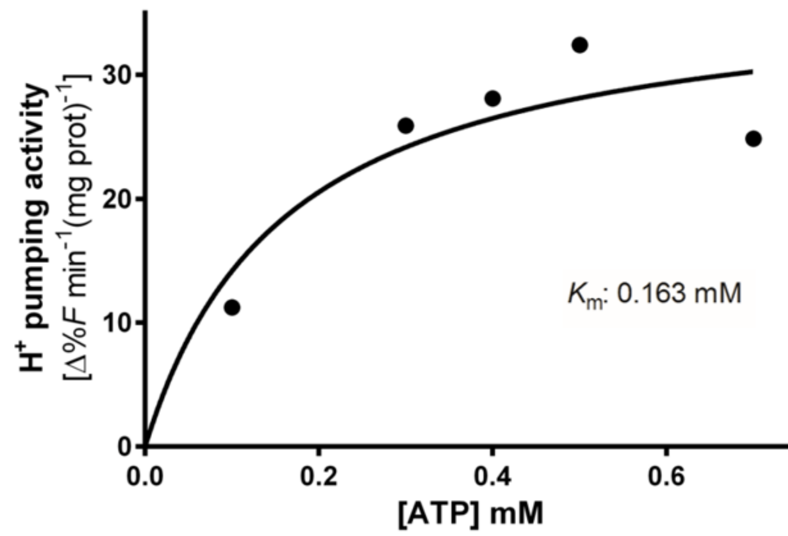


Figure IV.5: Proton pumping activity of V-ATPase in lysosomal fractions from rat liver. The accumulation of H^+ was determined by measuring the fluorescence quenching of ACMA, using an ATP concentration range of 0.1-0.7 mM. These results were obtained in collaboration with M. Gonçalves and L. Loureiro.