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Review Article

Perspective of the Use of Hamlet as Palliative in Patients Undergoing Chemotherapy

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Abstract

Cancer is the second cause of death worldwide, characterized by high and unregulated cell proliferation. The causes are multifactorial, including damage or mutation of proto-oncogenes, genetic predisposition and environmental factors. Various treatments have been used, such as surgery, radiation, chemotherapy and immunotherapy, with the aim of attacking mutated and highly proliferating tumor cells. Human Alpha-Lactalbumine (ALA) of molecular weight 14 kDa, is a small protein present in the human milk at a concentration of 2.0 mg/ml with lethal effects on cells of different types of cancer. It is commonly known as Human Alpha-Lactalbumine made lethal to tumor cells or HAMLET. The therapeutic efficacy of HAMLET has been demonstrated in animal models, with human glioblastoma xenograft, bladder cancer and bowel cancer *in vivo*. In clinical settings, HAMLET has been shown to act on skin tags. Cellular targets are found in the cytoplasmic membrane, cytoskeleton, mitochondria, proteasomes, lysosomes, and nuclei. In the mode of action of HAMLET it has been proposed that the protein insert in the cellular membrane and then internalize targeting the cytoplasmic membrane, cytoskeleton, mitochondria, proteasomes, lysosomes, and lastly nuclei. Inducing DNA fragmentation. By other hand, long term effect of Chemotherapy include symptoms such as fatigue, peripheral neuropathy, and cognitive impairment, whereas, late effects, including osteoporosis, heart failure, and secondary leukemia, occur after treatment ends, but are causally related to cancer and treatment exposures. Herein, we pinpointed the clinical use of HAMLET as palliative after chemotherapy treatment.

Keywords: Cancer; Human Maternal Milk; HAMLET; Chemotherapy; Radiotherapy; Surgery; Pharmacological Drugs

Introduction

Cancer is a multifactorial complex and intricate disease arising that has become among the most common causes of death worldwide and at serious public health problem [1]. It is characterized by overgrowth, proliferation leading to alterations in the immune system. The incidence of cancer is heterogeneous, and varies by geographical and economical region. For example, in developed countries, the most predominant types of cancer are lung, colon, breast, and prostate cancers; while in low and middle income countries predominate stomach, liver, esophagus, and cervix [1-3].

Different treatments exist as chemotherapy, surgery, radiotherapy, immunotherapy, and/r a combination of them [3:4]. For example, in the head and neck cancers, include surgery,

radiation therapy, medications, or a combination of the three [3]. However, the persistence in this is in the range of 19-59%, and a 10-year survival rate [4] but in other more severe cancers such as the cervix or the ovary is much less [1-4]. Even worst, the aforementioned treatment is that all possess numerous side effects and complications as has been described elsewhere, and the levels of efficacy oscillate between moderate to low in most of the cases [3]. Therefore, in cancer research the challenge is the understanding of the molecular and genetic basis of the formation and development of cancerous diseases, the development of safer and effective treatments that even delay their development or even reverse existing changes, and utmost allow early diagnosis [5]. On referring specifically to immunotherapy has become one of the most promising therapies available and that can attack several types of cancer. It has been pointed out that in the treatment of malignant neoplasm mostly predominate the use of interleukins since are responsible for the recruitment and signaling among anti-tumor cells. In fact, interleukins in addition to their pleiotropic action, can serve as initiators in the tissue immune response against tumor cells, therefore it has been considered as potential therapeutic agents in cancer treatment of malignant neoplasm due to the complexity and the interleukin signalization pathways as extracelular signal regulated kinase (ERK2), and c-Jun N-terminal kinase (JNK) [6,7], The hope and the potential reside in the possibility that can control and kill tumor cells by restauration of the immune system and with the less damage or side effects to the patients, using immune oncology, that have arisen as one of the most effective therapeutic strategies for cancer treatment [8-11]. One of them that has become very promising is cancer immunotherapy because may induce durable anti-tumor immune response. Nowadays, there are several of these, such as, -Immune checkpoints inhibitors;-Cancer Vaccines;-Oncolytic virus therapy;-T-cell therapy (adoptive cell), and;-Targeted monoclonal antibodies. Of note is are the several of these last compounds, such as PD-1/L1 inhibitors (discovered in 1990), CTLA-4 as ipilimumab, nivolumab, and atezolizumab have received approval from the Food and Drug Administration (FDA). By another hand, a breakthrough was the inception of the first chimeric antigen receptor (CAR). Thus, in 2012, a CAR-T cell therapy developed by Carl June successfully treated a patient afflicted with leukemia. Moreover, cancer vaccines, used and designed to stimulate the body's immune responses as a common antigen (Ag) can do it against tumor cells by utilizing tumor antigens, which for instance can lastly leads to the destruction of cancer cells [12]. As most of the vaccines, cancer vaccines can be either preventive or therapeutic. The current prophylactic cancer available and approved by the FDA are the HPV and HBV vaccines against some cancers. Whereas therapeutic cancer vaccines are specifically addressed to stimulate the body system's immune response against a specific tumor antigens. But it also can be used to manage tumor growth or even induce tumor regression. There are proposed mainly four types: a) Genetically engineered vaccines, b) tumor whole-cell vaccines, c) dendritic cell vaccines, and d) protein-peptide vaccines [8-12].

Featuring the alpha-lactalbumine (ALA) present in the human milk (HL)

Documented information that has suggested that human milk (HL) and breastfeeding are associated with protection against childhood cancer [13] including mothers who practice it [13], there are studies that report a decrease in the general incidence of childhood cancer, which occurs in infants fed with breast milk, in contrast to those who did not receive it, the protective effect has also been evaluated in relation to breastfeeding time >6 months [14]. This reported association has been sustained in subsequent studies [13,14]. The protection is based on many complex explanations; like the one you mention, that there are molecules in LH, with properties capable of influencing tissue development and maintaining homeostasis in the infant. On the other hand, during the research processes on adhesiveness, in lung cancer cells, a group from Lund Sweden used components of LH (casein) and identified a protein complex, which induced apoptosis in these cells, discriminating and respecting healthy cells [14] (Figure 1A). This complex was subsequently purified from LH casein, determining that the active component of the process fell on the protein; alpha-lactalbumin (ALA), which corresponds to one of the most abundant proteins of LH, whose approximate concentration is 2.0 mg/ml [14,15] (Figure 1A). They called the molecular complex with the lethal characteristics mentioned, derived from LH, HAMLET, for its acronym (human α-lactalbumin made lethal to tumor cells) whose particularity was to kill tumor cells, by leading them to a process similar to programmed cell death (apoptosis) [14,15] (Figure 1A). To elucidate the structure of the main protein of the complex, whey was used, identifying its crystalline structure. And for the purification of the apoptosis-inducing complex, a casein fraction of LH was used, and with the help of ion exchange chromatography, it

was determined that the eluate maintained its apoptotic activity. In it, proteins with molecular masses that varied from 14 to 100 KDa were identified, which showed homogeneity of the amino acids and their N-terminal sequences with the native ALA (Figure 1B). However, when testing with native ALA obtained from LH serum, no apoptotic activity was produced, which led to the assumption that the active form of ALA in the complex was different from native ALA [14,15] (Figure 1B) Indeed, it is known that ALA, under certain conditions of acidity, modifies its tertiary structure, which allows it to interact with lipids, since these act as cofactors that allow variants in protein folding, They can be stable and maintain a new conformation as lipid-protein complexes with diverse biological activity, as well as a function different from the original. Based on this knowledge, it has been determined that the apoptotic capacity of the complex; HAMLET, results from the folding of ALA and a lipid cofactor that, in this case, only C18 cis-unsaturated fatty acids are suitable for its formation [15]. Therefore, for the formation of the complex of; Human α-lactalbumin and oleic acid (C18:1:9 cis) with apoptotic capacity, a change in ALA in its structural form is necessary, since it must be partially unfolded to favor the formation of the complex [15,16].

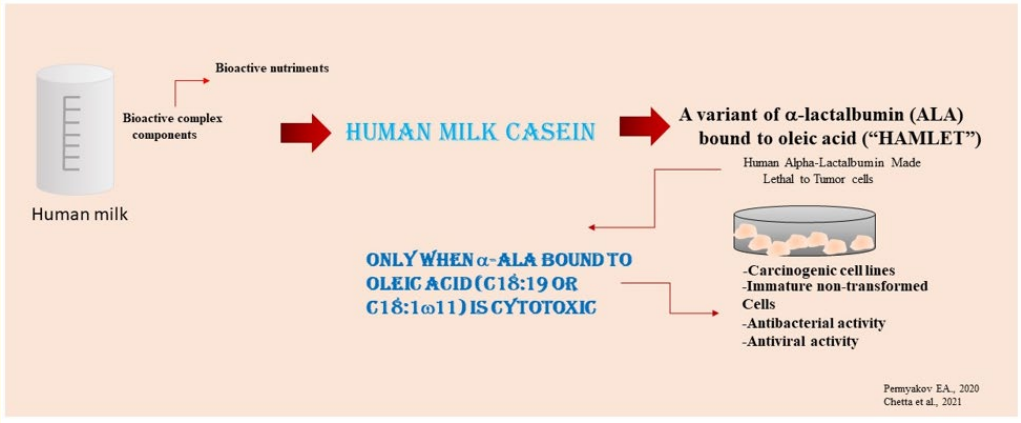

Figure 1A

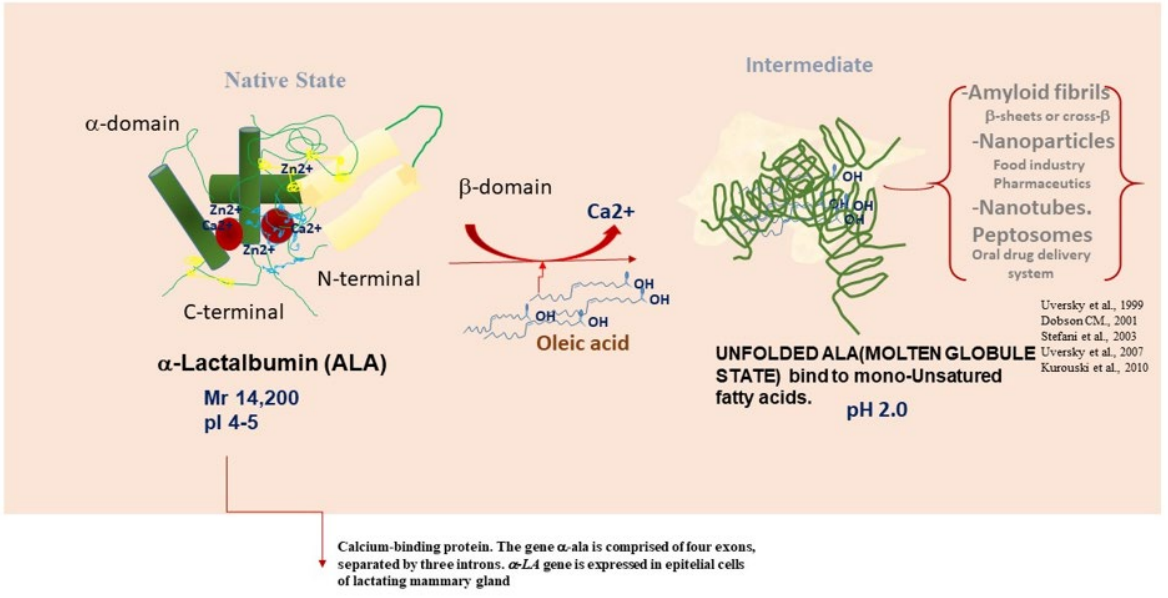

Figure 1B

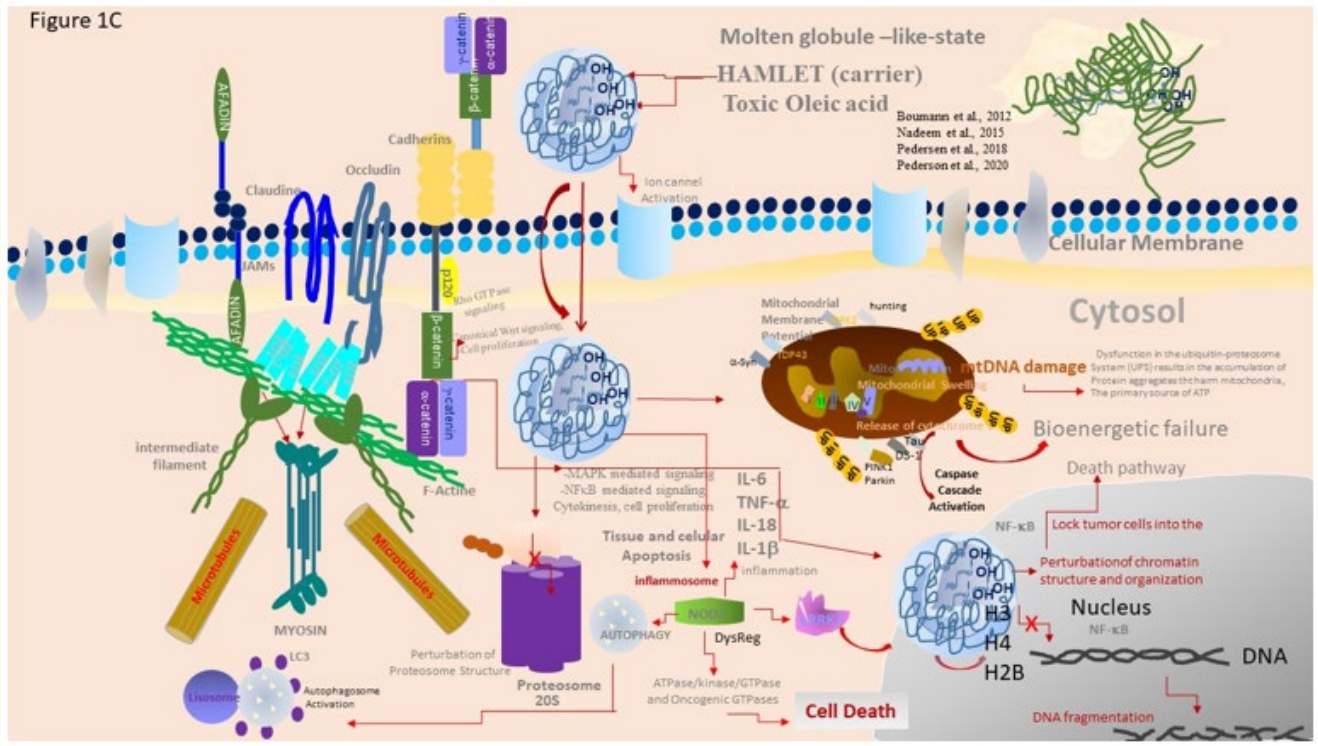

Figure 1. Schematic drawing of alpha-Lactalbumin (ALA) made lethal to tumor cells, named HAMLET (A). Native folded state and Calcium (Ca 2+) binding sites of ALA formed by alpha helices (n=3) and beta-sheets (n=2) and formation of protein lipid complex after releasing of calcium and oleic acid, forming a molten globule-like conformation. In this state, is able to form amyloid fibrils, protein aggregates of fibrillary morphology of 7 to 13 of diameter with a high increase in the beta sheet secondary structure. The phosphatidylserine a negative phospholipid present in the cellular membrane can induce protein fibrillation of a several proteins including alpha-LA, Thus, phosphatidylserine and other acid phospholipids can favor a physiological low pH environment enhancing protein fibrils in vivo. Indeed, it has proposed that phosphatidylserine-protein interaction can participate in the mechanism of cytotoxicity of the aggregated protein fibrils altering membrane function (B). The proposed mechanism of action of HAMLET as anti-tumorigenic complex of protein-lipid (like a core-shell, where the toxic oleic acid is the core surrounded by the alpha LA) is that under a state of molten globule (unfolded) can interact with the cellular membranes of tumor cells and internalize to the cytoplasm. The protein-lipid complex can activate several signalization pathways, such as MAPK(mitogen activated protein kinase), NF-κB(Nuclear factor kappa-beta), Caspase cascade, tissue and cellular apoptosis, autophagy, ionic channel activation, cell motility (myosin), Rho GTPase signaling, Canonical WnT signaling, Inflammation (production of pro-inflammatory cytokines), and Proteosome dysfunction, interaction with Nucleotide binding proteins (NOD2) involved in the activation of the autophagosome and fusion with lysosome. The protein-lipid complex also have an effect in the different cellular organelles, causing for example in the nuclei, hypodiploid DNa population,and DNA fragmentation (C).

The action mechanism of the complex lipid-protein (lipoprotide) alpha-lactalbumin-oleic acid like HAMLET

The question that has been the subject of intense investigation because the clinic implications is how the complex lipid-protein (alpha-LA-oleic acid) is cytotoxic. The mechanism of the cell penetration of the specific complexes of human alpha-lactalbumin (alpha-LA) with oleic acid (OA), HAMLET and LA-OA-17 (Zherolova et al., 2019) which possess cytotoxic activity against tumor cells. Therefore, to further approach and study the molecular mechanism underlying interaction of the OA complexes with cell membranes, with the small unillamellar dipalmitoylphosphatidycholoine (DPPC) vesicles and with electroexcitable plasma membrane of intermodal native and perfused cells of the green alga Chara corralling. It was found that OA-binding increases the affinity of alpha-LA to DPPC vesicles. Calcium association decreases protein affinity to the vesicles; the effect being less pronounced for A-OA 17. In addition, voltage clamp technique show that the LA-OA 17, HAMLET, and their constituents produce different modifying effects on the plasmalemmal ionic channels (Ca(2+) current and Ca (2+)-activated Cl(-) current and by increase in the nonspecific K(+) leakage currents of the Chara coralline cells. Thus, the modification of alpha-LA by OA results in an increase in the protein association with the model lipid bilayer and in drastic irreversible changes in permeability of several types of the plasmalemmal ionic channels. [17]. Moreover, the calcium ion binding decrease the affinity of the alpha-LA to the vesicles [17]. Moreover, Fang., *et al*. pointed out that for the anti-tumor activity of the HAMLET-like complexes of alpha-LA-oleic acid, also called lipoprotides, depends predominantly of the oleic acid while for the internalization, is the alpha-LA that is associated closely with the phagocytosis pathway [18]. Mossberg., *et al*. using fluorescence imaging experiments showed that this lipoprotein complex accumulates in the membranes vesicles, perturbing their structure and therefore in an enhanced membrane fluidity. Indeed it has been observed that at physiological conditons, snd neutral pH, the complex of HAMLET and oleic acid disrupts membrane integrity. Therefore, the proteinaceous alpha-LA component of the HAMLET complex lipoprotide is the ne that confers specificyt for tumor cells membrane precisely through protein interactions maintained by the oleic acid [19]. Moreover, ion fluxes are essential to initiate HAMLET-induced tumor cell death and to distinguish tumor cells from normal cells in this context [20]. In addition, and of note is that HAMLET in their molten globule-like state (unfolded state) form amyloid fibrils (Figure 1B-C) that can even form protein aggregates of fibrils of 7 to 13 nm with a higher beta-sheets content [21-23]. Protein misfolded can then form protein aggregates that under appropriate physiologic environmental conditions results in cytotoxic effects such as neurodegenerative disorders [24-27]. Furthermore, the protein misfolded can form lipoproteins (lipid and partially denatured proteins). On referring specifically to the alpha-LA with oleic acid, possess a common core-shell structure with cytotoxic effect [28]. Thus, the lipoproteins forming core-shell structures like micelles can be considered as molten globular containers filled with the toxic oleic acid. It is known that lipoproteins have the property of increase the fluidity of membranes. In this way, oleic acid can be transferred to vesicles. Therefore, the disruption of the plasma membrane is one of the major factors in the lipoproteins toxicity toward cancer cells [29]. This resembles a cargo off/carrier-loading of the oleic acid into cell membranes and internalization to the cytoplasm (Figure 1C). Therefore, the action of the molten globule-like state of the alpha-LA complexed with oleic acid is favored by the interactions of the proteinaceous shell with the acidic phospholipids present in the cellular membranes and internalization to the cytoplasm [30] (Figure 1C).

After the initial selective recognition of the alpha-La-oleic acid, Ho., *et al*. suggested that one of the targets of this complex was the nucleotide-binding proteins. Moreover, the authors proposed that the dysregulation of the ATPase(GTPase machinery contributes to cell death [31], and the tumoricidal effect of the complex of alpha-LA-oleic acid (HAMLET) may be based on dysregulation of kinases and oncogenic GTPases, a process to which tumor cells are addicted. Furthermore, using proteomics analysis of the tumor cells treated with alpha-LA –oleic acid (288), identified 112 differentially expressed proteins, of which 95 were upregulated that satisfy the metabolism of tumor cells; 17 were downregulated and targeting alpha-La-oleic acid. These data allow to conclude that alpha-LA oleic acid anti-tumor activity is through disruption of the cytoskeleton stability and cell motility [31-33] (Figure 1C). Whereas the inhibition of DNA, lipid, and adenosine triphosphate (ATP) synthesis, results in cellular stress and therefore in the activation of programmed cell death [49,50]. HeLa cells treated with alpha-LA-oleic acid leads to oxidative stress and activation of apoptosis [34,35] because this stimulation may induce a condition

in which the ATP production exceeds the energy demand, Moreover, alpha-LA-oleic acid (HAMLET) organized and structured as core-shell lipoproteins may also exert several and a diverse affects in the different cellular organelles, that includes mitochondrion, lysosome, nuclei, cytoskeleton [34,35] (Figure 1C). From the cytoplasm to the nuclei, it has been found that the complex is associated with histones H3, H4 and H2B [36,37] in tumor cells, perturbing the chromatin structure, and impairing their deposition on DNA. The consequence of this is that locks the cells into the death pathway [38]. Furthermore, the complex multimeric of alpha-LA-Oleic acid induced a loss of the mitochondrial membrane potential, mitochondrial swelling, and the release of cytochrome c (Figure 1C) followed by activation of the caspase cascade [39,40]. Interestingly, the treatment of RAW264.7 cells (macrophages cell line from a mouse tumor induced with Abelson murine leukemia virus) with a high concentration of alpha-LA (> or = to 100 µg/ml) caused morphological changes and a dose time dependent increase in growth activity. As aforementioned above, the effects of the complex alpha-LA with oleic acid induced at nuclei, an increase in DNA fragmentation and a hypodiploid DNA population [41]. At this high dose of alpha-LA-oleic acid induces apoptosis, necrosis and enhanced expresso of cytochrome c, active caspase 3, caspase 8, and of the signalization pathways, extracellular signal regulated kinase (ERK2) and c-Jun N-terminal kinase (JNK) (Figure 1C) without affecting protein levels [41]. Indeed, inhibition experiments with the broad spectrum inhibitor of cell death, Boc-D-fmk Boc-D-fmk, failed to block it, showing thus, that alpha-LA-oleic acid induced cell death is modulated through a caspase-independent way. Furthermore, it has been reported that in mice, the complex alpha-LA inhibits the development of colon carcinogenesis. This effect can be due to a decrease in the prostaglandin E2 production given cyclooxygenase-2 inhibition by Alpha-LA [42]. Because cyclooxygenase is expressed in colon carcinogenesis and plays a role in the disease progress. Therefore, long term consumption of the complex alpha-LA-oleic acid reduces colon carcinogenesis progression of the disease. In addition to their anti tumorigenic activities, the complex protein-lipid possess antibacterial and antiviral properties especially toward resistance strains to antibiotics [43,44]. Of note is that alpha-LA is endowed with immunomodulatory capabilities as a native or unfolded state. It has direct effects on B and T lymphocytes, enhancing for example antibody response to systemic antigens [44].

Perspective of complex protein-lipid, "HAMLET"

There are studies that conclude that the partial deployment of ALA is necessary, but not sufficient to kill the tumor cell, therefore, this property would be determined by the participation of; the unfolded protein and the lipid cofactor C18:1:9 cis [44]. In the studies to clarify the molecular mechanisms of the formation and stabilization of HAMLET, there was a specific one carried out to convert native ALA to a partially unfolded variant with biological function altered [45] (Figure 1B). Due to certain processes, proteins adopt different conformations, either due to their amino acid sequence or thermodynamics itself, which forces the protein to modify itself with the lowest free energy. However, although this classic view has been questioned because it has been seen, that proteins adopt their shape according to their kinetic pathway [45]. The HAMLET protein ALA has a shape or state similar to the molten globule (MG), which is an intermediate folding state [46,47]. ALA of LH has its native protein conformation while remaining in the mammary gland environment as part of the "synthase complex," required in the production of lactose [47]. However, the protein can undergo folding modifications under certain conditions as; change in the environment, in breastfeeding and the digestion process, or in manual expression to preserve it and use it later, and in some cases donate it to a Human Milk Bank (BLH). If the latter is the case, the milk must be preserved frozen -21°C, and pasteurized to provide it to a recipient (hospitalized baby). This process in Mexico is carried out according to the official Mexican standard (NOM) [48]. Well, under these storage conditions and LH procedures, ALA can initiate a change in its conformation, which would potentially form bioactive complexes such as HAMLET [49,50]. This complex has been used and has begun to appear in new therapies, in our case, since we have two BLH, in two hospitals, one in the Zacatecana Woman (HMZ) and another in the Fresnillo hospital (HGF), both from the State of Zacatecas, Mexico, we have tested its benefits in patients undergoing chemotherapy and radiotherapy, as palliatives for the side effects of these therapies, whose results have been encouraging [51]. Therefore, based on the properties of HAMLET and particularly ALA in the form of MG, it is possible to propose its use as a nutracenic element, within the palliative care of patients undergoing chemotherapy, by virtue of having been used by more than one year, in at least ten patients, who voluntarily participate in an uncontrolled clinical trial, approved by the Research Ethics Committee of the Zacatecas General Hospital, "Luz González

Cosío", Mexico, who have reported a notable improvement in their condolences and effects. Because these outstanding empirical clinical observations, our group is currently focused to determine the patterns of gene expression before and after oral administration to patients with different types of cancer.

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Author's Statement

The authors declare not conflict of interest.

Author's Contribution

A.A.C. and G.G.G.M. Conceptualization, writing and reviewing.

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