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# Human milk as a source of tumor killing molecules. From MAL to HAMLET.

Mossberg, Anki

2010

Link to publication

*Citation for published version (APA):*
Mossberg, A. (2010). *Human milk as a source of tumor killing molecules. From MAL to HAMLET.* [Doctoral Thesis (compilation), Division of Microbiology, Immunology and Glycobiology - MIG]. Inst of Laboratory Medicine, MIG.

*Total number of authors:*
1

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# Human milk as a source of tumor killing molecules

From MAL to HAMLET

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Akademisk avhandling
som med vederbörligt tillstånd från Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Rune Grubbsalen, BMC, Sölvegatan 21, Lund
Fredagen den 19 mars, 2010, kl 13.00

Handledare:
Professor Catharina Svanborg

Fakultetsopponent:
Professor Lawrence J. Berliner
Department of Chemistry and Biochemistry,
University of Denver, USA

| Organization<br>LUND UNIVERSITY<br><br>Faculty of Medicine<br>Institute for Laboratorymedicin<br>Department of Microbiology, Immunology and Glycobiology (MIG) | Document name<br>DOCTORAL DISSERTATION<br><br>Date of issue March 19th 2010<br><br>Sponsoring organization |
|---|---|
| Author(s)<br>Ann-Kristin Mossberg | |


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Title and subtitle

Human milk is a source of tumor killing molecules From MAL to HAMLET


Abstract

HAMLET (Human alpha lactalbumin made lethal to tumor cells), a complex between the partially unfolded alpha lactalbumin and oleic acid, was discovered by serendipity when anti-adhesive properties of human milk were examined HAMLET kills tumor cells but not healthy differentiated cells.
Native alpha lactalbumin (HLA) can be conveted to HAMLET by a two-step process including: 1) calcium ion removal inducing a teritary change of the protein structure 2) incorporation of oleic acid into the apo-protein by an ion-exchange chromatography.
Alphalactalbumin from several species can be converted to HAMLET like complexes The tumor killing complex is present in human casein after low pH preicipitation in contrast to animal-casein The results suggest that the alphalactalbumin protein from different species fulfill the properties for HAMLET formation but oleic acid is not accessible in the animal caseins.
HAMLET interaction with membranes was investigated as a mechanism for HAMLET uptake HAMLET interacts and pertubes the structure of membrane vesicle and induce leakage of small molecules as well as morphological changes but there was no evidence of HAMLET uptake into the vesicles.
The HAMLET activity was investigated in vivo. Patients with bladder cancer were subjected to daily intravesicle HAMLET instillations. Massive tumor cell shedding was detected into urine after each instillation and a decrease in tumor volume was detected at surgery HAMLET had a tumor selective activity in human patients as there were no detectable signs of cell death in the healthy tissue of the bladder. The therapeutically value of HAMLET was further investigated in an orthotopic mouse model. Tumor area in HAMLET treated animals were significant reduced compare to controls. Whole body imaging showed retention of HAMLET in tumor bearing tissue as Alexa-labelled HAMLET was visualized in the bladder 4 hours after the instillation.
In summery we show that human milk is a source for tumor killing molecules We described the composition and the conditions for HAMLET formation and we also shows evidence for therapeutically value in vivo.



Key words: Protein folding, alpha lactalbumin, human milk,

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language: English

ISSN and key title: 1652-8220 Human milk as a scource of tumor killing molecules

ISBN: 978-91-86443-34-4

Recipient's notes

Number of pages

Price

Security classification


Distribution by (name and address)


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# Human milk as a source of tumor killing molecules

From MAL to HAMLET

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Department of Microbiology, Immunology and Glycobiology

2010

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Back side cover: Alphalactalbumin purification by HIC
Front side cover: HAMLET conversion
Blue line shows conductivity
Brown line shows absorption at 280nm

Printed by Tryckeriet E-huset, Lund 2010

ISBN 978-91-86443-34-4
ISSN 1652-8220

*Skrutten &*
*Snäckan*

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## List of papers

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**I. Svensson M, Hakansson A, Mossberg A-K, Linse S and Svanborg C.** (2000) Convesion of alpha-lactalbumin to a protein inducing apoptosis. *Proc natl Acad Sci USA* 97, 4221-6

**II. Pettersson J, Mossberg A-K and Svanborg, C.** (2006) Alpha-Lactalbumin species variation, HAMLET formation and tumor cell death.
*Biochemical and Biophysical Research Communications* 345:260-270.

**III. Mossberg A-K, Puchades M, Halskau O, Bauman A, Lanekoff I, Chao Y, Martinez A, Svanborg C and Karlsson R** (2010) HAMLET interacts with lipid membranes and perturbs their structure and integrity. *PLoS One* (Published February 23rd, 2010)

**IV. Mossberg A-K, Wullt B, Gustafsson L, Månsson W, Ljunggren E and Svanborg C.** (2007) Bladder cancers respond to intravesical instillations of HAMLET. *Int. J. Cancer*: 121, 1352-1359

**V. Mossberg A-K, Hou Y, Svensson M, Holmqvist B and Svanborg C.** (2009) HAMLET treatment delays bladder cancer development. *Journal of Urology* (accepted for publication, April 2010)

## Abbreviations

| | |
|---|---|
| aa | amino acid |
| ANS | 8-anilinonaphtalene-1-sulfonic acid |
| BAMLET | bovine alpha-lactalbumin made lethal to tumor cells |
| BCG | Bacille Calmette-Guerin |
| BSSL | Bile salt stimmulated lipase |
| C18:1 9 cis | oleic acid |
| CIS | cancer in situ |
| CD | circular dichroism |
| ER | endoplasmatic reticulum |
| ERAD | ER-associated degradation |
| FFA | free fatty acid |
| GMP | Good Manufacturing Practice |
| GT | galactosyltransferase |
| GUV | giant unilamellar vesicle |
| HAMLET | human alphalacalbumin made lethal to tumor cells |
| HIC | hydrophobic interaction chromatography |
| HLA | human alphalactalbumin |
| LUV | large unilamellar vesicle |
| MAL | multimeric alphalactalbumin |
| MRI | magnetic resonance imaging |
| NMR | nuclear magnetic resonance |
| PI | propidium iodine |
| PBS | phophate-buffered saline |
| PFA | paraformaldehyde |
| PMV | plasma membrane vesicle |
| PS | phosphatidyl serine |
| $rHLA^{All\text{-}Ala}$ | cystein to alanin mutant of human alphalactalbumin |
| SPR | surface plasmon resonance |
| TCC | transitional cell carcinoma |
| TUNEL | terminal deoxynuclotidyl transferase biotin-dUTP nick end labeling |
| UPR | unfolded protein response |
| UV | ultraviolet |
| WBI | whole body imaging |

## Summary

HAMLET was discovered by serendipity when anti-adhesive properties of human milk were examined. When casein was mixed with tumor cells, the cells died and showed signs of apoptosis. The active casein fraction was shown to contain the whey protein alphalactalbumin, but native alphalactalbumin, was not killing tumor cells. The protein in the active fraction differed from native alphalactalbumin by being partially unfolded, as shown by Near UV CD spectra and an increased ANS binding. In paper I we show that partially un-folded alphalactalbumin forms the active complex by binding a co-factor, oleic acid (OA). We also defined the two-step process required to convert purified native alphalactalbumin to a complex with oleic acid, showing the same tumor killing activity as the active fraction from human milk. In step one, the calcium ion in native alphalactalbumin, is removed by EDTA treatment, resulting in a partial un-folding of the protein to the apo-state. In step two, the oleic acid is incorporated into the apo-protein by ion-exchange chromatography and the complex is eluted with high salt buffer. The findings defined HAMLET as a new molecular entity with tumoricidal activity.

In paper II we investigated how protein sequence variation influences HAMLET-like complex formation. The amino acid sequence of alphalactalbumin is highly conserved but varies approximately 25-30% between the human protein and proteins purified from cow, horse, pig or goat. Unlike human, the animal milk casein showed no tumor killing activity after low pH precipitation. However, purified alphalactalbumin, from all species could form active complexes by the two-step conversion method defined in paper I, suggesting that the sequence variations between alphalactalbumins from different species does not change their ability to form HAMLET.

In paper III we investigated the HAMLET interaction with artificial phospholipid membranes or vesicular membranes from tumor cells. We show that HAMLET interacts and perturbs the structure of different membrane vesicle preparations. Leakage of small molecules from the internal of membrane vesicles was detected when vesicles were subjected to HAMLET. There was no evidence of HAMLET uptake into the vesicles. Tumor plasma membrane vesicles (PMV) were prepared and mixed with Alexa-labeled

HAMLET. A dotted binding pattern indicating selective HAMLET binding to certain membrane areas on the PMV surface but there was no evidence of translocation across the membrane. We conclude that HAMLET binds to and interact with lipid membranes in the absence of specific protein receptors and that the uptake depends on cellular components not present in the artificial vesicle preparations. These interactions are important to understand the differences in HAMLET uptake between tumor cells and healthy cells, a key step in the tumoricidal process.

In paper IV, the effect of HAMLET on human bladder cancers was examined. Patients with bladder cancer were subjected to daily intravesical HAMLET instillations during the week before scheduled surgery. Urine samples were collected before and after each of the instillations and photographs of the papillary tumors were obtained before and after the instillation period. We detected massive shedding of tumor cells into the urine after each HAMLET instillations. TUNEL assay showed the presence of HAMLET induced DNA fragmentation in shed cells as well as in biopsies from remaining tumors. In contrast, there was no sign of tissue damage in biopsies from healthy areas of the bladders. Eight out of nine patients showed a macroscopically detectable decreases in tumor volume or a reduction in biopsies positive for *CIS* (cancer in situ). We conclude that HAMLET showed a tumor selective activity in human patients.

In paper V the therapeutic value of HAMLET was further investigated in an orthotopic mouse bladder cancer model. Tumors were established and then treated with five intravesical instillations of HAMLET or PBS. The tumor size was determined by histology and cell death by TUNEL staining. We show that the tumor size was significantly reduced in HAMLET treated animals compared to PBS controls. We also show, by whole body imaging (WBI) that the uptake and retention of HAMLET was specific for tumor tissue and Alexa-labeled HAMLET was detected in the bladders of tumor bearing mice but not in healthy bladders. We conclude that HAMLET has a therapeutic potential by killing tumor cells *in vivo* and thereby delaying tumor progression in the mouse model.
In summary, we show that human milk is a source of tumoricidal molecules such as the HAMLET complex. We define HAMLET as a complex between alphalactalbumin and oleic acid, clarify the conditions for HAMLET formation and show evidence of therapeutic activity *in vivo* in humans and experimental mouse model.

## Svensk populärvetenskaplig sammanfattning

Via bröstmjölken får barnet inte bara i sig näringsämnen utan även andra komponenter som kan skydda barnet från infektioner och kanske även från allergier och cancer.

HAMLET (**H**uman **a**lphalactalbumin **m**ade **le**thal to **t**umor cells) är ett komplex bestående av vassleproteinet alphalactalbumin och en fri fettsyra (oljesyra). Komplexets förmåga att döda cancer celler men inte friska celler upptäcktes av en slump när bröstmjölk delades upp i kasein och vassle i jakten på anti bakteriella komponenter. I de arbeten som presenteras i avhandlingen visar vi hur vi utifrån inaktiva komponenter kan skapa HAMLET komplexet med dess nya celldödande egenskaper.

Vi visar även att alphalactalbumin framrenat från andra arter (ko, häst gris och get) kan användas för att bilda HAMLET liknande komplex. Men, eftersom effekten saknas i kaseinet, drar vi slutsatsen att den nödvändiga fettsyran inte är tillgänglig för komplexbildning i mjölken från dessa djur.

Vi har på olika sätt visat att HAMLET tas upp av cellerna och interagerar med olika cellulära delar och strukturer. Upptaget till cellerna verkar vara avgörande för om cellerna är känsliga eller resistenta för HAMLET-inducerad celldöd. Vi har sett att friska celler inte tar upp något HAMLET, medan tumörceller inte bara tar upp HAMLET utan även transporterar det hela vägen in i cellkärnan. Mekanismen bakom upptaget är fortfarande inte klarlagt. Sedan tidigare är det känt att alphalactalbumin kan binda till cellmembranliknande ytor och vi undersöker i ett arbete om HAMLET har kvar denna egenskap samt om det kan vara en bidragande faktor till det cellulära upptaget. I studierna använder vi protein som är märkt med en fluorokrom, vilket gör att vi med hjälp av fluoroscensmikroskopi kan titta på inbindningen till artificiella membraner. Studierna visar att HAMLET binder till membranen, men vi kunde inte upptäcka något upptag till det inre av lipidmembran vesiklarna.

I arbete IV och V undersöker vi den tumörcellsdödande effekten *in vivo*, dels på patienter med cancer i urinblåsan och dels i en mus-modell, där mössen har fått tumörceller inplanterade i urinblåsan. I båda fallen visar vi att HAMLET har effekt på tumörcellerna. Patienterna fick, i väntan på operation, dagliga installationer av HAMLET-lösning (5 dagar), via kateter in i blåsan. Fotografier på tumörerna tagna före och efter installationsperioden, visade på tydliga skillnader i storlek och utseende. Vi detekterade också döda tumörceller i urinen efter varje installation, vilket indikerande

att HAMLET påverkade tumörerna i blåsan på patienten. Vi kunde dock inte se någon påverkan på den friska vävnaden i blåsan. För att kunna fortsätta utreda den tumördödande effekten av HAMLET behövde vi utveckla en djurmodell. Efter de positiva resultaten från blåstumörstudien i människa ansåg vi att denna tumörform var önskvärd även för fortsatta studier. Det är sedan tidigare känt att HAMLET inaktiveras i blod och därför är de intravesikulära (via kateter in i blåsan) instillationerna en bra modell för att undvika inaktivering. Det är också ett vanligt distributionssätt för andra läkemedel vid behandling av blåscancer.

Vi visar i arbete V att tumörceller som installeras i blåsan på sövda möss, snabbt etablerar sig och växer ut till solida tumörer. Vi visar också att HAMLET instillationer minskar tillväxthastigheten på tumörerna och i vissa fall också undviker tumörbildningen helt. Fluoroscens märkt HAMLET kunde också påvisas, med hjälp av ”whole body imaging”, i urinblåsan med tumörer en lång tid (flera timmar) efter själva instillationen

Sammanfattningsvis visar de inkluderade arbetena att det finns cancercelldödande komponenter i human mjölk. Vi visar vad dessa komponenter består av och hur vi kan ta fram dessa komponenter från human mjölk.  HAMLET dödar många typer av tumörceller i laboratoriemiljö och vi har visat att HAMLET även har denna effekt på tumörer i patienter och i möss.

# Introduction

## Background

When breastfeeding, the mother does not just provide the baby with nutrients but also with a range of other molecules beneficial for the child. Epidemiological studies have shown that breastfeeding protects against infections in the gastrointestinal-, respiratory- and urinary tracts. The most studied and best-described effecter molecule is secretory IgA, protecting the child from disease during early childhood when the immune system is poorly developed. Lactoferrin, lysozyme, lactoperoxidase, anti-bacterial peptides and complement factors are also present in milk. Together these components may lyse bacteria, inhibit growth and may thus contribute to the anti-infective defenses in milk [3,4,5] recently reviewed by [6]. For both viruses and bacteria, attachment is essential to get in close contact with the mucosal cells. Binding facilitates infection but also prevents bacteria from being washed away or caught and eliminated in the mucus covering the epithelial cell layer [7]. Human milk contains a variety of carbohydrates that can act as anti-adhesive molecules, by binding to viral and bacterial adhesion molecules preventing them from attaching to the epithelial cells of the host [8]. Secretory IgA can also have an anti-adhesive effect [9].

The HAMLET project started during a study of *Streptococcus pneumonieae* and *Heamophilus influenzae* attachment to epithelial cells. The aims were to identify molecules responsible for adherence and to correlate anti-bacterial and anti-adhesive properties of milk to colonization and infection. In a clinical study, we obtained nasopharyngeal bacterial cultures at 2, 6 and 10 month of age from children. Milk from their mothers was collected at the same times. Strains from episodes of disease (otitis, sinusitis or pneumonia) were also collected. The IgA antibody levels in the milk, specific to pneumococcal capsular types (6A, 19A and 23F), to type 3 polysaccaride, to C-polysaccaride and to phosphorylcholine (PC) were compared to the types of bacteria found in the child. The episodes and severity of disease as well as asymptomatic carriage were also correlated to the antibody findings. Unexpectedly, no protective role of the different antibodies studied were detected either to carriage or disease [10].

Bacterial adherence was then quantified using human epithelial cells, scraped from nasopharynx of healthy donors and scored by interference contrast microscopy [11]. To study the inhibition of bacterial adherence by components in milk, human whole milk or milk fractions were introduced in the adhesion assay, and inhibition was quantified. Substantial inhibition of the binding was detected with the casein fraction [12] and this effect was resistant to both trypsin- and heat- treatment and was thought to originate from the heavily glycosylated κ-casein.

Virus infections have for a long time been suggested to promote secondary bacterial infections in the upper respiratory tract [13]. To further address the relevance of viral infection for attachment, we established a viral/bacterial super-infection model. We replaced the primary cells with a human lung carcinoma cell line, which could be infected by viruses typical for the respiratory tract and displayed receptors for bacterial binding. The attachment of *S. pneumonieae* to those cells was increased after the virus infection [14], and human breast milk, or fractions from it, inhibited this increased attachment.

The casein from human milk, did not just affect bacterial attachment, but also killed the cancer cells. By light microscopy we observed that the cell morphology changed and cell death was confirmed by trypan blue exclusion. The cells were smaller, the nuclei more condensed and in some cases also fragmented (figure 1) when stained with propidium iodine (PI). These morphological changes suggested that the cells might die by apoptosis or apoptosis like cell death and this was further supported by DNA fragmentation resulting in 50 kbp fragments and DNA-laddering [15]. We concluded that the casein fraction from milk must contain a component, which is toxic to tumor cells. As healthy cells in primary culture were not killed, we also concluded that tumor cells were more sensitive than healthy differentiated cells.

**Figure 1**

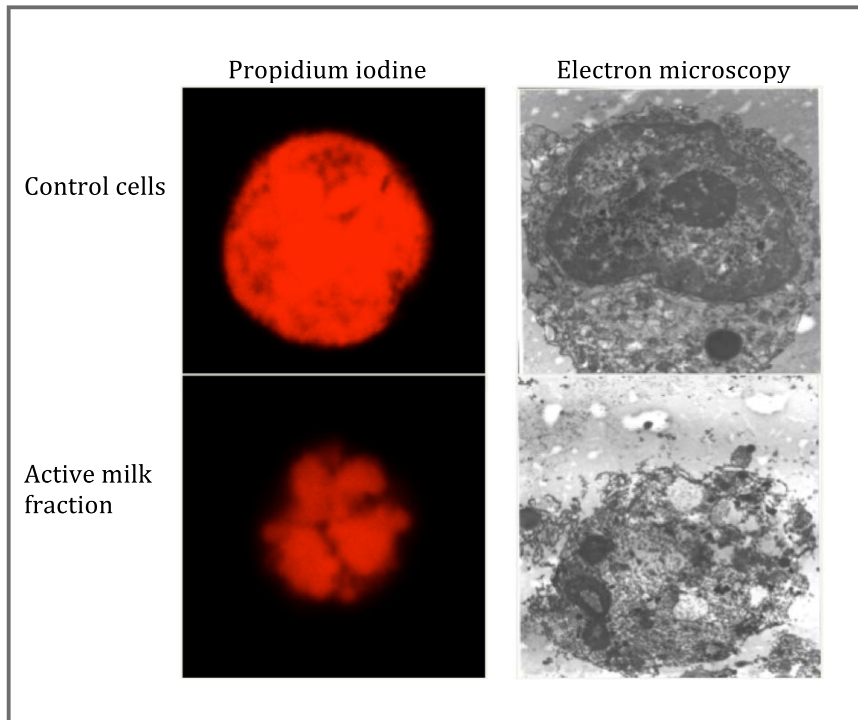



**Figure 1.** Left panels. PI staining of tumor cell nuclei Uniform stain of control cell nuclei compared to the nuclei of cells exposed to the active milk fraction.
Right panels. Electron microscopy of tumor cell nuclei after exposure to human milk shows disintegration with apoptotic body formation

## Casein and MAL

Casein is a mixture of milk proteins precipitating at pH 4.3(human) or 4.6 (bovine) [16] and makes up about 80% of the bovine but only about 35 % of the human milk proteins [17]. The casein appears as large micelle like aggregates containing 450-10 000 subunits[18]. The bovine casein fraction contains α- (s1) and (s2) caseins, β-casein and κ-casein, all rich in prolines and phosphate group or serine residues of importance for micelle formation. The high concentration of proline residues inhibits the formation of close-packed secondary structures and the conformations of the proteins are much like denatured globular proteins and do not contain any disulphide bonds. The human casein can also be divided into β (phosphorylated)-and κ (glycosylated)-casein or subgroups of the two. The biological role of casein is not totally elucidated but the amino acid sequence gives a slow but steady release of amino acids adjusted to the need of the child. The casein micelles carry large amounts of calcium and phosphor and the micelles can also contain citrate, ions, lipase and other enzymes to provide a more efficient nutrition (reviewed in [6]).

To isolate the active component in the casein the precipitate was subjected to ion exchange chromatography according to Kunz and Lönnerdal [16]. Five different fractions were eluted with a sodium salt gradient and the effect on tumor cells was tested. Neither of the separated fractions was tumoricidal and the activity was not recovered after mixing of the fractions. We realized that the active component might still be attached to the column and added an additional washing-step with high salt buffer (1M NaCl that released a sixth (VI) protein fraction (figure 2). After dialysis and lyophilization the fraction was shown to induce cell death [19].

The major protein component in fraction VI was a 14 kDa protein visible on SDS gels but with presumed multimeric forms with a size of about 28 and 50 kDa. The proteins in each of those SDS bands were identified, by Edman degradation, as the whey protein alphalactalbumin (HLA) [15]. The active fraction, fraction VI, was called MAL (**M**ultimeric **A**lpha-**L**actalbumin) due to its tendency to show multimeric forms on SDS PAGE gels.

**Figure 2**

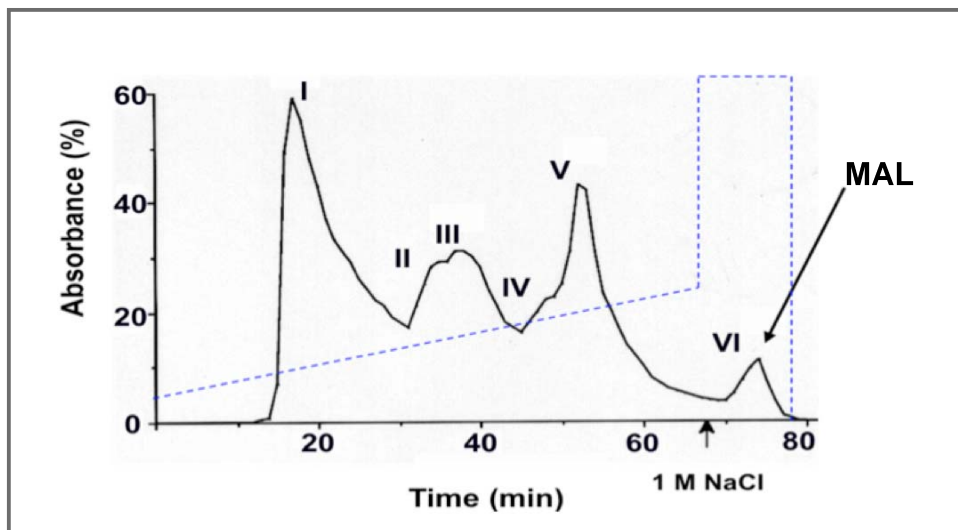



**Figure 2.** Ion exchange chromatography showing the elution of casein fractions I-V with a salt gradient (0-30% NaCl). An additional washing step (1M of NaCl) released fraction ,VI, MAL.

HLA, is a whey protein but has an isoelectric point close to 4.6, and is known to co-precipitate with casein [20]. However, alphalactalbumin, purchased from a commercial source or purified from human milk using hydrophobic interaction chromatography (HIC), did not kill the tumor cells even at high concentrations [15].

After those experiments, three different areas of investigation were defined; 1) *What is MAL? Structure and composition of the active fraction in human casein.* 2) *Why are the cells dying? Cellular mechanisms.* 3*) Can the cell killing activity be used therapeutically?* This thesis is based on papers dealing with each of those basic questions and covers the time from to the discovery until today.

## AIMS

To study the

**I. The chemical composition/identity of the tumoricidal component in the casein fraction VI.** Can the activity be reproduced from single chemically defined components?

**II. if HAMLET formation is conserved among lactating animal species?** Influence of protein sequence variation and fatty acid composition/availability.

**III. if HAMLET interacts with artificial membranes**. Binding, perturbation and effects on membrane integrity.

**IV. if HAMLET kills human tumor cells *in vivo*,** when topically administered to cancer patients? A study of bladder cancer.

**V. if HAMLET is therapeutic,** in a murine bladder cancer model.

## MAL becomes HAMLET

### Paper I. Conversion of α-lactalbumin to a protein inducing cell death

The protein in fraction VI, the MAL fraction, was shown to be human alphalactalbumin (HLA). HLA is a globular milk protein of 123 amino acids and with the molecular weight of 14.2 kDa. The protein is ubiquitous and exclusively expressed in secretary cells of the lactating mammary gland [21,22]. The protein is, after folding in the ER, transported to the Golgi apparatus and acts there as a substrate specifyer of galactosyltransferase and promotes lactose production. The protein is also secreted into the milk and is present in high concentrations during the whole lactation period. Alphalactalbumin consists of two domains held together by one of four disulphide bounds present in the protein (aa73-91). The α-helical-domain contains 3 major α-helices (aa 5-11, 23-34 and 86-98) and two short $3_{10}$-helices. The smaller β-sheet-domain consists of a triple-stranded anti-parallel β-sheet (aa 40-50). Three additional disulphide bounds are located within the α-helical-domain (aa 6-120, 28-111) and in the β-sheet-domain (aa 61-77) (Figure 3) [1].

**Figure 3.**

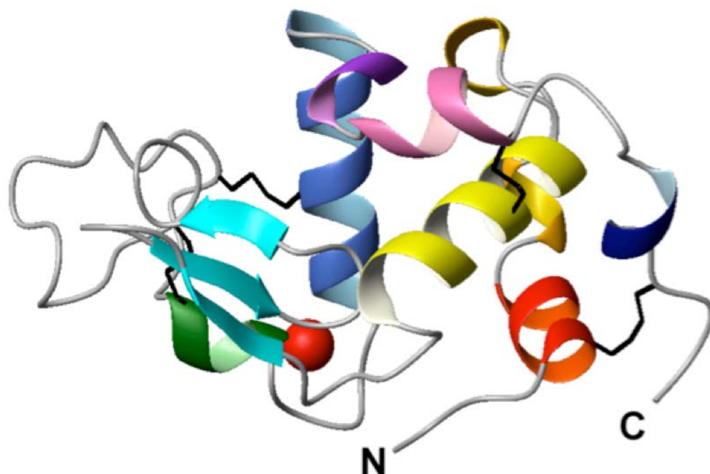



**Figure 3.** Schematic of native alphalactalbumin. The α-helical (right)- and the β-sheet (left)- domain are linked by a strong calcium binding site (red sphere). Two intra-domain disulphide bonds (black) are present in the α-helical domain, one in the β domain and one is linking the two domains together [1].

HLA purified from whey by hydrophobic interaction chromatography (HIC) was not tumoricidal and no post-translational modifications of the protein in the active fraction were detected [19]. This suggested that the difference in activity were either due to the

purification method or to structural variation. When the calcium ion, coordinated by the side chains of Asp 82, 84, 87 and 88 as well as Lys79 [1], is removed the protein forms partly un-folded intermediates. Low pH, as in casein precipitation, induces the molten globule state characterized by native like secondary structure but lack of the specific tertiary packing of the side chains and exposes hydrophobic surfaces. (table 1) [23]. The partially un-folding of the protein is reversible and in physiological environments ($Ca^{2+}$, temperature or pH) the protein returns to native state, reviewed in [23].

Changes in tertiary structure can be detected by the Near UV CD spectroscopy reflecting the environment of tyrosines and tryptophans. The spectrum of a well-folded protein is distinct in contrast to the spectra of a molten globule where the loss of signal indicating less ordered side chains. ANS is a hydrophobic dye that binds to hydrophobic surfaces and increases in fluorescence and a shift to shorter wavelength is observed when ANS is bound. CD spectroscopic analysis, and ANS spectra indicated that the HLA present in MAL had altered tertiary structure, resembling a molten globe [19].

**Table 1.**

| Name | Inducer | Features |
|---|---|---|
| A-form | Low pH | "Classical molten globule". Partially unfolded but with native-like secondary. |
| Apo-state | Chelators / EDTA | Removal of calcium ion. Show the most distinct CD spectra among the partially unfolded forms. |
| T-form | heat | CD spectra close to the A-form |
| P-form | Low concentration of denaturans | CD spectra close to the A-form |

**Table 1.** Folding states of alphalactalbumin, and conditions leading to their formation [23].

To prove the hypothesis that a change in tertiary structure accounted for the activity of MAL we decided to deliberately unfold native HLA and test its tumoricidal activity.

Native alphalactalbumin, purified from whey, was unfolded by EDTA treatment (apo-state), and subjected to ion-exchange chromatography as described above. Despite the EDTA treatment the protein eluted in the void volume, showed native state signals by CD spectroscopy and was inactive when tested on tumor cells. However, when the procedure was repeated using a matrix pre-conditioned with casein, the protein was retained on the matrix, only eluting with high salt. After desalting and lyophilization the eluted fraction killed tumor cells. This suggested that the active complex must contain a

1,0

co-factor from casein that stabilized the alternative fold of the protein and conferrers activity.

To identify the co-factor we analyzed casein components on the matrix previously used for MAL purification. No proteins other than alphalactalbumin were detected, but milk lipids were bound to the column matrix. The yield of active fraction was gradually reduced when re-using the casein-conditioned matrix and repeatedly adding apo-alphalactalbumin. The results indicated consumption of a cofactor. To identify this cofactor matrix samples were collected after the first, fifth and tenth run and subjected to different extraction protocols including chloroform/methanol/water. TLC separation of the organic phase revealed differences in free fatty acid (FFA) content, indicating a gradual depletion of FFA from the matrix.

The FFAs present in casein were identified as 16:0, 14:0 and 18:1 by GC/MS (Gas Chromatography/Mass Spectroscopy). To examine which of those FFAs could form a complex with alphalactalbumin, pure fatty acids were purchased, individually applied to new matrix and subjected to apo-alphalactalbumin. Only the matrix pre-conditioned with oleic acid (18:1 9cis) was showed to retain the protein and to form a tumor-cell killing complex (figure 3). The results suggested that the active complex contained alphalactalbumin in a partially unfolded state bound to oleic acid.

**Figure 3**

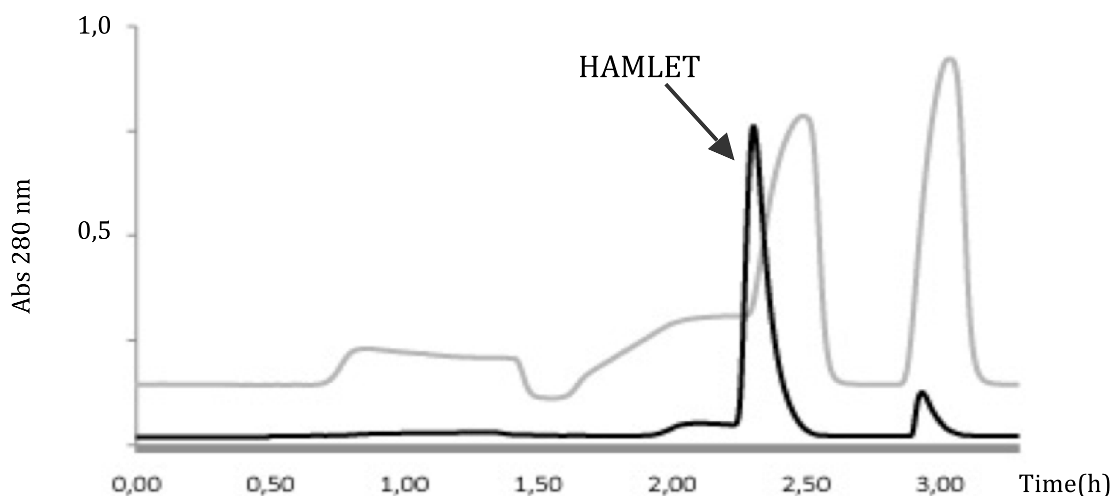



**Figure 3**. HAMLET conversion on an oleic acid conditioned matrix. Early shift in conductivity (grey line 0.8 -1,4 h) indicates EDTA in sample buffer. The converted complex elutes after addition of high salt buffer (black line, peak at arrow).

To exclude contaminant from human milk, recombinant alphalactalbumin was expressed in *E. coli*, treated with EDTA and subjected to ion exchange chromatography on an oleic acid conditioned matrix. The elution pattern resembled the one seen with whey-derived protein and the recombinant protein-lipid complex showed the same cytotoxic activity. After near UV CD spectra, ANS binding, cell death analysis and NMR studies, we concluded that the complex between apo-alphalactalbumin and oleic acid, formed on the ion-exchange column matrix were identical with fraction VI isolated from casein. This protein-lipid complex was named HAMLET (**H**uman **A**lphalactalbumin **M**ade **LE**thal to **T**umor cells).

The stereo-specific protein-lipid interaction between human alphalactalbumin and the fatty acids was later further investigated. In human milk the triglycerides contain mainly long, unsaturated fatty acids such as oleic acid. In contrast, short saturated fatty acids are found in cows milk [24]. A range of fatty acids, differing in carbon chain length, saturation and cis- or trans orientation, was used [25] and complexes were analyzed by near UV CD spectroscopy, ANS binding, cell death inducing activity and NMR. Complexes were formed by ion-exchange chromatography, with all long chained cis- unsaturated fattys acid tested (18:1 9 cis, C18:1 11 cis, C16:1 9cis, C20:1 11 cis, C18:3 6,9,12 cis, C18:1 6 cis, C18:2 9,12 cis, C20:4 5,8,11,15 and C18:3 9,12,15 cis). However, only complexes with unsaturated 18 carbon chain fatty acids induced cell death. The mono-unsaturated 9 *cis* (oleic acid) and 11 *cis* (vaccenic acid) showed strongest activity indicating a stereo-specific interaction between the protein, the fatty acid and the tumor cells. None of the fatty acids induced cell death when mixed with tumor cells at concentrations present in HAMLET complex [25].

To examine if folding change makes alphalactalbumin cytotoxic we used recombinant bovine protein mutants, locked in the un-folded state by point-mutations in the calcium binding site (D87A [26] and D87N [27]). We showed that partial un-folding of the protein was not enough to induce tumor cell death but both proteins, though, could readily be converted to HAMLET like complexes with similar activity [28]. The need for the fatty acid for activity has later been confirmed using the HLA$^{All\text{-}Ala}$ mutant [29]. This recombinant mutant protein is unable to form disulphide bonds since each of the cysteins is replaced by alanin [30]. As a result, HLA $^{All\text{-}Ala}$ is not able to reach the native

state, as confirmed by CD spectra and NMR [31]. We showed that the protein alone did not trigger cell death. HLA $^{All-Ala}$ formed an active complex without EDTA pre-treatment, indicating that a partially un-folded structure able to bind to oleic acid [29].

### Paper II. α-Lactalbumin species variation, HAMLET formation and tumor cell death.

The alphalactalbumin and lysozyme genes originate from the same ancestral gene. After gen duplication, 300-400 million years ago, the genes still share about 40% identity [32,33]. The alphalactalbumin protein is present in all mammalian milks and is highly conserved throughout evolution. The bovine, caprine, equine and porcine protein sequences show about 70% identity and more than 80% homology to human alphalactalbumin and the calcium-binding region is 100% conserved. The aim of paper II was to elucidate if the natural alphalactalbumin sequence variants, found in different species, altered the ability to form HAMLET like complexes 1) directly from casein or 2) when exposed to oleic acid in the conversion protocol [34].
Casein protein were obtained from human, cow, horse and pig milk according to Melander, O ( Uppsala Läkareförenings Förhandlingar 1947), briefly milk was defatted by centrifugation and diluted in deionized water to double volume. Hydrochloric acid was slowly added to a final pH of 4.6. The milk was allowed to precipitate at 4°C over night and the casein was extensively washed and lyophilized. Alphalactalbumin was also purified from human, cow, horse, goat and pig milk using hydrophobic interaction chromatography [35].

The tumoricidal activity of the different caseins was examined. Only human casein killed the tumor cells and the difference in HAMLET-like-complexes content was confirmed by ion-exchange chromatography showing very low yield in all but the human casein. However, when casein was subjected to conversion on oleic acid conditioned matrix, active complexes were formed. This indicates that the free fatty acid (oleic acid) content in the animal casein was to low for complex formation.
The purified alphalactalbumin proteins were subjected to conversion according to paper I. The species variants were readily converted to complexes with tumoricidal activity resembling HAMLET although the yield of formed complexes differed, being highest

with human- and lowest using porcine protein. The results show that the sequence variations do not of prevents alphalactalbumin to form HAMLET like complexes.

Milk from different animals differs in fatty acid composition. In human milk oleic acid is the most abundant fatty acid in the tri-glycerides. Other fatty acids dominate in other species. The fatty acids are released by lipases during digestion in the intestine. Bile-salt-stimulated-lipase (BSSL) is an enzyme, which hydrolyzes the ester bond between glycerol and the lipids, enabling the fatty acids to be released. The lipase is excreted from the pancreas but has also been found in human milk [36] allowing free fatty acids to be released before reaching the pancreatic enzymes. Differences in fatty acid composition as well as presence of BSSL might thus explain why HAMLET like complexes is formed only in human milk casein.

## HAMLET Production

To support ongoing research, HAMLET is continuously produced according to the two-step process established in paper I, using human milk as the source of HLA. In Sweden breastfeeding is encouraged. Large hospitals also have a routine of collecting milk from mothers with a surplus to supply premature babies. Milk, stored more than 3 months, cannot be used as food and is normally discarded. This milk and milk from donors in late phase of the lactation is collected in collaboration with the University Hospital in Lund and used for HAMLET production. The milk is kept frozen at -20°C until use.

The HAMLET production method can be divided into three major steps: 1) *HLA purification*, 2) *Conversion* and 3) *Dialysis and lyophilization*. The process can be visualized schematically as shown in figure 4.

*Human Alphalactalbumin (HLA) purification*

The HLA concentration in milk is stable over time and between donors (about 2g/L). After thawing, the milk is processed to remove tri-glycerides (centrifugation) and large unrelated proteins (ammonium sulphate precipitation). HLA is purified by hydrophobic interaction chromatography (HIC) according to [35]. In brief, hydrophobic surfaces are exposed in HLA when the calcium ion is removed by EDTA. The apo-protein binds to the HIC-matrix and is released in a defined fraction as calcium ions in the elution buffer revert the protein to the native state. This process can be scaled up and is only limited

by matrix capacity and column size. The protein is further purified by size exclusion chromatography and dialysis or used directly for conversion (figure 4).

*Pre-conditioning*

Prior conversion the matrix is pre-conditioned with oleic acid. The anion exchange matrix, DEAE Trisacryl M, was used when MAL was discovered. Despite several attempts, no other commercially available matrix have been shown to be superior and this matrix is therefore still in use.

The molecular interactions that bind the fatty acid to the matrix are not fully understood. The matrix is positively charged and at pH 8.5, the pH used for conversion, oleic acid is de-protionated giving a negative net charge. The carboxyl-group of the fatty acid can then bind to the matrix, leaving the hydrophobic tails facing the water phase.

Interesting, the fatty acid is not released from the matrix in respond to high conductivity (data not published) if not bound to alphalactalbumin, contradicting a regular ion-exchange binding.

The dispersion of hydrophobic compounds, such as fatty acids, in water, is incomplete. For pre-conditioning of the matrix, oleic acid is purchased at a purity of >95% and dispersed in small volume of ethanol before an emulsion in buffer is obtained by mixing. The emulsion is passed over the column matrix, allowing the fatty acid to bind. The top layer of the matrix (0-1 cm) contains, by far, most of the fatty acids, shown using radio labeled oleic acid (data not published). This suggests that the height of the ion-exchange column is of less significance for the conversion yield compared to the area of the matrix bed.

The molar ratio (protein: lipid) has been determined as 1 : 4-8 by acid hydrolysis and GC/MS but the HAMLET preparation might be a mixture of complexes containing different numbers of lipid molecules. To avoid a lipid overload, e.g. adding more lipids to the protein than necessary for the activity, the production is balanced with a protein surplus giving less than 100% of conversion yield.

*Conversion*

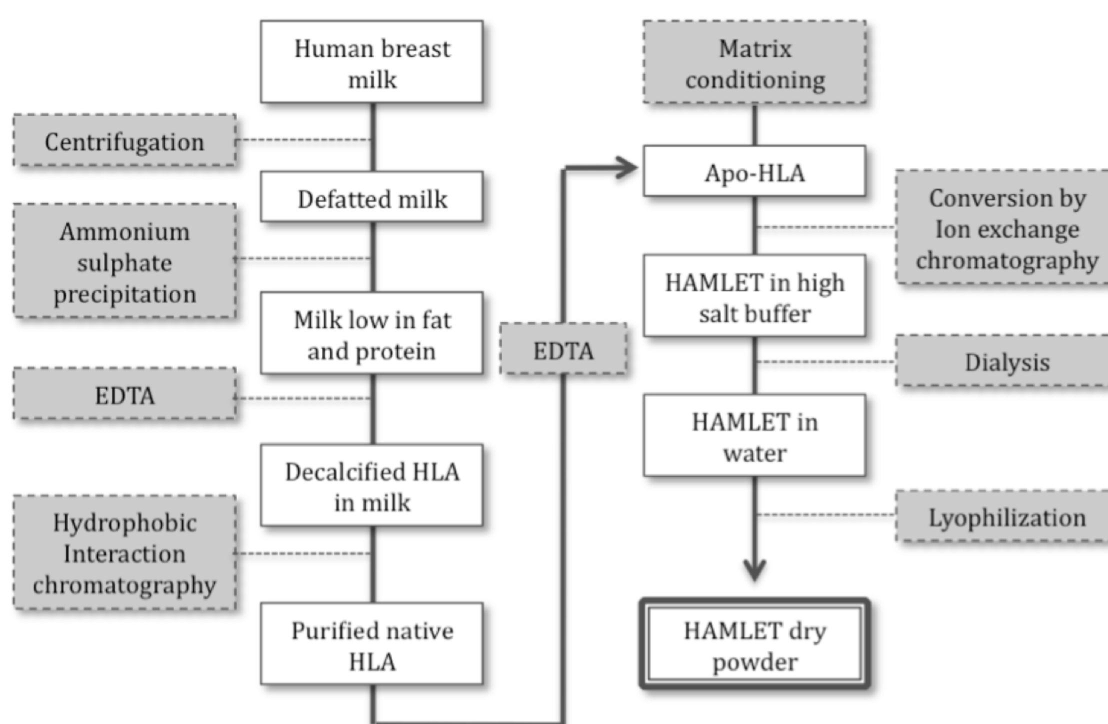
In the conversion process, un-folding of HLA is achieved by EDTA treatment (apo-form) but HLA in molten globule state (low pH) has also, successfully been used for conversion. EDTA is a chelator binding divalent anions with high affinity but also acts as a salt and increases the conductivity of the solution. In HAMLET production, high conductivity prevents the binding between matrix/lipid/protein and the EDTA

concentration has to be balanced not to interfere with the conversion yield.

*Dialysis and lyophilization*

The final production step is removal of salt and buffer by dialysis against de-ionized water. Extensive water volumes are needed and the dialysis process is time consuming. It cannot be excluded that a prolonged dialysis might affect the HAMLET complexes and possibly reduce or enhance the HAMLET activity. However it has not been possible to replace the dialysis step in the laboratory scale production. The protein is subsequently lyophilized. Stability studies have shown that lyophilized HAMLET maintains its activity for several years at -20°C.

**Figure 4. Schematic of HAMLET production**




*GMP and large scale production*

The production, described above, is an ongoing process within the HAMLET project. The capacity is a weekly production of one gram of HAMLET consuming about 1 to 1.5L of human milk. To supply material for further *in vivo* studies in humans, HAMLET has to be produced according to GMP (Good Manufacturing Practice) regulations. In collaboration with Biovian (Åbo, Finland) the HAMLET production protocol has successfully been transferred and used for GMP production.

Within the tech transfer process, each step of the process was analyzed and critical parameters identified. Criteria and accepted tolerances levels for the incoming and outgoing product were established as well as analytical methods for each of the steps. Unexpectedly, human milk is regarded as a pure toxin until otherwise proved.

Tech transfer to GMP accredited biotech facility uncovered new parameters such as matrix stability, virus clearance validation, fill-finish and stability studies, to be considered in a cost efficient HAMLET production. However, the tech transfer also proved the stability and reproducibility of the process developed in the research laboratory environment.

## Cellular responses to HAMLET

The early studies of Håkansson et al [37], showed that MAL binds to the surface of tumor cells and, to a lesser extent, to healthy human renal tubular epithelial cells (HRTEC). In tumor cells the protein was also detected in the cytoplasm and the nuclei, but internalization was not seen in healthy cells. The differences were also confirmed by sub-cellular fractionation with $^{125}$I-labelled MAL. After 1 hour a large proportion of the recovered radioactivity was detected in the nuclear fraction of both A549 and Jurkat cells and over time, nuclear accumulation increased. Only a small fraction of the radioactivity added to the healthy cells was detected in the cell fractions and mainly located in the cytosolic fraction [37]. The uptake of MAL to different cell compartments was later verified using Alexa-labeled HAMLET showing specific binding to histone proteins [38]. The difference in sensibility between the tumor cells and healthy cells was therefore assumed to be connected the differences in uptake [37].

### Paper III. HAMLET interacts with lipid membranes and perturbs their structure and integrity.

Alphalactalbumin is known to interact with lipid bilayers and the interaction is dependent on the folding state of the protein and the lipid composition of the membranes [39]. In paper III we investigated if HAMLET retained the ability to interact with lipid bilayers using artificial lipid vesicles and if this binding could facilitate HAMLET uptake to cell compartment. Artificial large unilaminar vesicles (LUV) and GUV (Giant unilaminar vesicles) were formed from lipid film, hydrated in required buffers

[40,41]. To examine if HAMLET binds cell surface membrane, we used membranes originating from tumor cells (Plasma membrane vesicles PMV, prepared according to [42]).
SPR (surface plasmon resonance) was used to measure the interaction between liposomes and HAMLET. We found that HAMLET associated with the three different LUVs used but native HLA was only binding to liposomes at non-physiological pH and only to membranes with negative net charge. The binding of HAMLET to GUVs, made from both egg yolk and soybean, was verified by fluorescent microscopy using Alexa Fluor labeled proteins. Neither native HLA, rHLA$^{\text{All-Ala}}$ nor the Alexa dye alone bound to the membranes as strongly as HAMLET. When mixing HAMLET with liposomes (LUV), filled with fluorescent ANTS and its quencher DPX, leakage of the liposome content in response to HAMLET was detected. Native HLA did not cause leakage under physiological conditions. We also observed morphological changes of the GUV membranes at different time-points after HAMLET treatment. The membranes appeared to be elongated, thinner and more flexible compared to GUVs treated with HLA or rHLA$^{\text{All-ALA}}$.

To examine the interaction of HAMLET with tumor cell PMVs, bleb formation was initiated by DTT and aldehyde treatment [42]. Alexa-labeled HAMLET was shown by confocal microscopy, to bind PMV membranes but we could not detect uptake of HAMLET into the vesicles. HAMLET binding to the PMV differed to the binding we previously detected to EYPC or Soybean vesicles in that the binding was not homogenous but accumulated in distinct membrane dots. Native HLA or Alexa dye alone did not bind to in the membrane bleb.
We concluded that HAMLET interacts with phospholipid membranes under physiological conditions. This binding differs from the earlier observed pH dependent binding of HLA and is not just due to the conformational change of HLA. HAMLET binding destabilizes and causes morphological change of plasma membranes and can be facilitated by the direct interact showed by binding to LUV and GUV. It is though possible that the dotted binding to PMV indicates binding to receptors or to certain structures of the plasma membrane with enhanced binding capacity. The lack of uptake to the vesicles indicates that other cellular components, like the cytoskeleton, are required for the translocalization of HAMLET across cellular membranes.

**HAMLET and Apoptosis**

It was previously shown that tumor cells show signs of apoptosis such as cell shrinkage, DNA condensation and fragmentation after exposure to MAL/HAMLET (Figure 1). The continued studies now suggest that HAMLET triggers several cellular events and different types of programmed cell death. Some of the results are summarized below.

The ability to induce cell death in response to different stimuli is essential to prevent cells from uncontrolled cell growth. Apoptosis and autophagy are key cell death responses and are vital to all organisms. A considerable number of diseases are due to errors in these processes [43,44] reviewed in [45].

Several pro- and anti- apoptotic genes are critical in tumor cell development, such as *Bcl-2* [46] and *p53* [44]. Mutations in cell death signaling pathways can facilitate malignant transformation, due to uncontrolled proliferation without the need for growth factors [47]. Oncogenic mutations also reduce the sensitivity to internal DNA damage [48], reviewed in[49]. Apoptosis can be initiated both via intrinsic as well as extrinsic mechanisms, reviewed by [50] (figure 5). The Fas ligand is as a well-known signal transductor of extrinsic apoptotic signaling pathway. The activation of caspase 8, via the Fas ligand system activates downstream caspases leading to cell death. The activation of caspase 8 can also activate the intrinsic apoptosis pathway via Bid (a pro-apoptotic member of the Bcl-2 family) leading to cytochrome c release from the mitochondria [51]. The Fas receptor was excluded in the HAMLET induced cell death when antagonistic antibodies to CD95 (Fas receptor) did not show any protective effect [52].

The intrinsic apoptotic pathway can, for example, be activated by DNA damage. Bax is released from its cytoplasmic anchor and the Bax homodimer or Bax/Bak heterodimer interact with the voltage-dependent anion channel of the mitochondria leading to the release of cytochrome c [53]. The release of cytochrome c results in the formation of the apoptosome including the pro-caspase 9 and apoptosis activation factor 1 (AAF1)(figure 5). Activation of caspase 9 and 3 leads subsequently to the cell surface exposure of PS

(Phosophatidyl serine), DNA condensation, DNA cleavage, apoptotic body formation and cell death. Caspase activation and cytochrome c release are hallmarks of classical apoptosis. By using zVAD-fmk, a pan caspase inhibitor, PS exposure and the caspase dependent nuclear condensation were inhibited in HAMLET treated cells but no rescue from cell death was observed [52,54].

The Bcl-2 family includes both pro- and anti-apoptotic proteins [55]. Bcl-2 and Bcl-XL, belongs to the latter group, and block the release of cytochrome c from the mitochondria. Bcl-2 or Bcl-xL over-expression makes cells less sensitive to apoptosis inducing stimuli, as the balance between pro- and anti-apoptotic proteins is disturbed [56]. No differences to HAMLET activity were detected when cells over-expressing Bcl-2 or Bcl-xl were compared to cells with physiological expression levels [54].

**Figure 5**

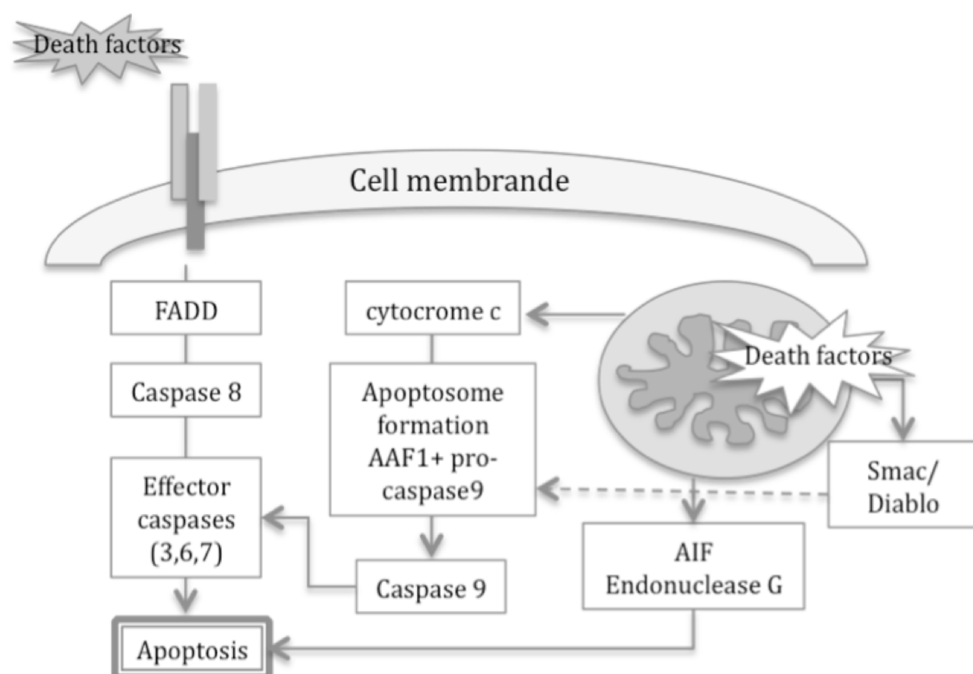



**Figure 5**. Simplified schematic figure of pathways leading to apoptotic cell death. The extrinsic pathway via the CD95 receptor and activation of caspase 8 leads to the cleavage of effector caspases. The intrinsic pathways converge at the mitochondria and include cytochrome c and AIF release leading to apoptosome formation. Small molecules, such as Smac and Diablo are also released to prevent apoptosis inhibitors from stop the apoptotic process [2].

We conclude that HAMLET probably induce cell death in several different ways and the observed signs of apoptosis (PS exposure, DNA condensation and fragmentation) is not originating from the initial trigger of cell death rather a secondary response from a dying cell.

## HAMLET and Autophagy

Autophagy is regarded both as a cell survival and as a cell death mechanism and is highly conserved through evolution. At low nutrient access, cells use autophagy to recycle cellular organelles and proteins, thereby prolonging cell survival and delaying or inhibiting apoptosis [57,58]. Autophagy can also be lethal to cells and cause programmed cell death; type II, initiated by stimuli such as prolonged starvation and mitochondrial damage (figure 6). Autophagy takes part in the immune response against infections caused by viruses and other intracellular parasites (resently reviewed by [59]) and defects in this signal pathway may lead to tumor development [60].
The apoptosis and autophagic pathways are also “cross-talking” at different levels [61,62]. There are several examples were a defect in the apoptotic system leads to activation of the autophagic cell death machinery and also examples showing an increase in apoptotic characteristics when autophagic cell death response is defect or blocked by different inhibitors [62]. Autophagy is characterized by autophagosome formation [63]. Whole organelles such as mitochondria, proteasomes and ribosomes can be enclosed within the autophagosome and digested by enzymes from fusing lysosomes.
One characteristic of autophagy is the modification of LC3-I to LC3-II, which translocates to the autophagosome. The translocation can be inhibited by 3MA an inhibitor of the class I phospahitdyloisitol 3-kinase (PI 3-kinase). PI 3-kinase class III, in complex with Beclin-1, a part of the complex responsible for autophagic vesicle nucleation [64].

HAMLET triggers autophagosome formation in lung carcinoma cells [65] as shown by transfection with LC3-GFP plasmid. When the transformed cells were treated with HAMLET there was a change, from the green florescent LC3-I protein uniform staining to a LC3-II granular pattern. The change in LC3 pattern was also observed in MDA-MB-231 breast carcinoma cells stably transfected with LC3-GFP. This granular staining was reduced by 3MA [65]. It was also shown that the Beclin-1 (mRNA as well as protein)

levels were increased after HAMLET treatment indicating that the autophagy machinery is responding to the HAMLET complex.

**Figure 6**

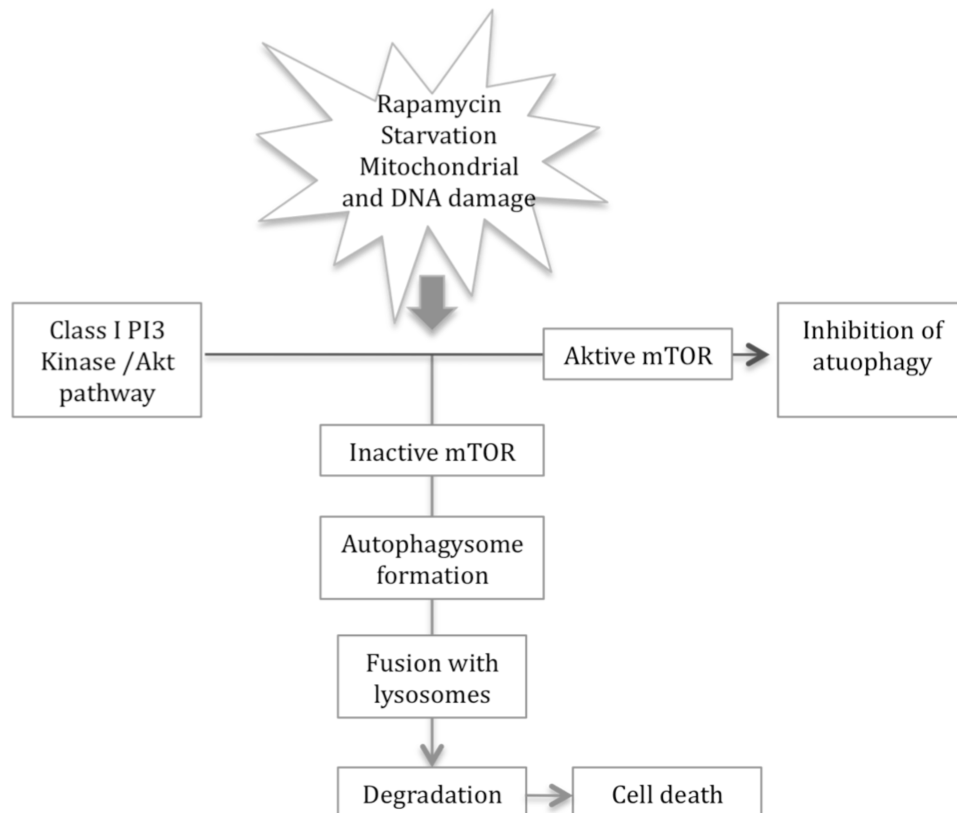



**Figure 6**. Simplistic figure of autophagy initiation. At normal nutrient condition mTOR is activated via PI3K-I and AKT leading to cell growth and proliferation. In case of starvation or mTOR inhibition by rapamycin the autophagy program is activated leading to autophagosome formation and degradation. Prolonged mTOR inactivation can lead to cell death.

## ER stress

The endoplasmatic reticulum (ER) is designed to synthesized and fold nascent membrane proteins and proteins destined for the Golgi apparatus. Unsaturated fatty acids are synthesized in the ER as well as cholesterol to enable new lipid membrane formation. ER is also the intracellular calcium store and is playing an important role in the calcium homeostasis. When either of the ER functions is perturbed, the ability to properly fold proteins will be compromised leading to a activation of a stress signaling program (reviewed in [66]). The PERK kinase is one of the first sensors to recognize the

accumulation of unfolded proteins in the ER. By phosphorylation of the translation initiation factor eIF2a, the protein synthesis is slowed down to give the ER time to regain function. If the accumulation of unfolded protein is prolonged ATF6 will enhance the production of ER chaperones for more efficient folding of the accumulated proteins. The third step to restore normal ER function is the activation of IRE1 leading to up regulation of proteins involved in the ERAD (ER associated degradation) system [67]. Unless unfolded protein load is reduced, signals will lead to cell death via apoptosis (reviewed in [68] or authophagy [69]) (figure 7).

Ongoing work indicates that all three ER stress sensors are activated by HAMLET treatment (Petter Storm submitted manuscript). A rapid increase in phosphorylated eIF2a, cleavage of ATF6 and mRNA splicing of XBP1 occurred within 30 minutes after HAMLET treatment. Finally, CHOP, a pro-apoptotic transcription factor strongly linked to ER stress was shown to be up regulated in response to HAMLET.

**Figure 7**

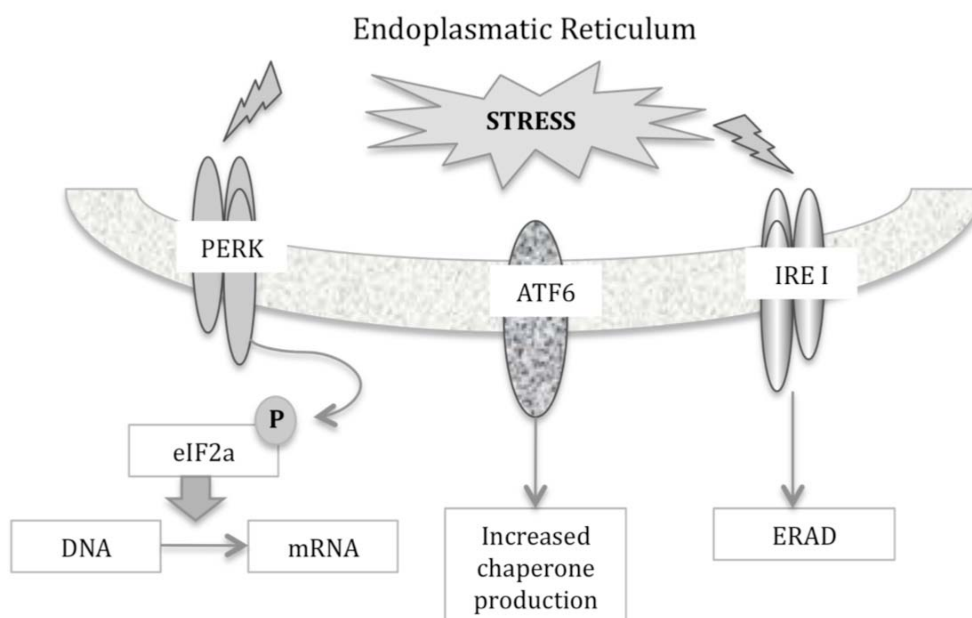



**Figure 7.** Accumulation of un-folded proteins in the ER is recognized by sensor proteins. PERK phosphorylates the transcription factor eIF2a to prevent further protein production giving time for ER to adjust the overload. Prolonged ER stress leads to ATF6 and IRE1 activation and if the stress situation is not solved the cell will die by apoptosis or autophagy.

### Proteasome overload.

Mammalian cells use two ways to degrade intracellular proteins. The autophagy machinery uses the proteolytic enzymes within the lysosomes after fusion with the autophagosomes. Proteins failing to fold in the cytoplasm are addressed for ubiquitin-proteasomes degradation [70]. The ERAD (ER associated degradation) system removes unfolded proteins from ER in response to stress and subsequently addresses the proteins for proteasome degradation. During cell cycle the cell up- and down-regulates protein expression. To stop signals or return to a normal homeostasis the proteins has to be removed and degradation is carried out by the proteasomes [71].
The 20S proteasome contains 2 α-and 2 β-subunits were the β-subunits holds the proteolytic activity and the α-subunits unwinds and guide unfolded proteins into the proteolytic core. In the 30S proteasome the β-subunits are guarded by a 19S subunits, which binds to the poly-ubiquitin and feeds the protein into the proteasome, while hydrolyzing the ubiquitin subunit. Summarized by [72].
To our knowledge HAMLET is not ubiquitinated in tumor cells even though it is an unfolded protein and should be subjected to proteasomes degradation. The 20S proteasome does not need the ubiquitin signal for protein degradation, however and HAMLET targets 20S proteasome or the subunits and inhibits the proteasome activity in isolated proteasomes as well as in intact cells*.* HAMLET also disrupt proteasome structure as shown by a change in molecular size of the different subunits [73]. In tumor cells, large quantities of miss- folded proteins are produced and they are destined for degradation by the proteasomes. In this situation a reduction in proteasome capacity, due to HAMLET inhibition or destruction might further promote cell death.

## HAMLET in vivo

### Paper IV. Bladder cancers respond to intravesicle instillations of HAMLET.

Tumor cell lines, originating from different species and tissues, are susceptible to HAMLET induced cell death [74]. This broad, tumoricidal activity makes HAMLET an interesting new drug candidate.

In a *xenograf* rat model of human glioblastoma, tumor cells were isolated from patient biopsies and cultured as spheroids *in vitro*. Subsequently spheroids were injected into the striatum of immuno-deficient rats [75]. The tumor cells were allowed to establish in the brain of the rats for 1 week and then subjected to a 24 hour infusion of HAMLET or native HLA, 0.7 μmol by convection enhanced-delivery into the xenografted area. The results showed that HAMLET treatment delayed the onset of symptoms ($p<0.001$) compared to HLA [75]. Using the TUNEL assay it was shown that HAMLET caused DNA damage only in the tumor area. $^{125}I$- labeled HAMLET was also shown to be distributed throughout the whole hemisphere.

The first *in vivo* study in humans was performed in patients with skin papillomas (warts) [76] which are benign tumors formed by HPV (human papilloma virus) transformed keratinocytes. Immune-suppressed patients run an increased risk of papilloma infections and [77] multiple wart formation. The risk of developing cervical cancer from HPV infection has been confirmed and vaccines against HPV serotypes that cause cervical infections are now offered to young females [78]. The 40 patients, included in the study, had papillomas, previously shown to be resistant to conventional treatment. They were enrolled in a randomized, placebo-controlled, double blind study [76]. HAMLET or placebo was applied topically on to each of the lesions daily during 3 weeks and covered with hydrocolloid dressing (Comfeel). The lesion volumes were measured and photographically recorded every week during the treatment and also one and two month after completed treatment. The randomization code was broken one month after all patients had completed the 3 weeks of treatment. All lesions treated with HAMLET showed a reduced volume and 88 out of the 92 were reduced $\geq$ 75%. The median volume after treatment was 14% of the starting volume. In the placebo group only 15 out of 74 lesions showed a 75% volume reduction. This study showed that HAMLET has therapeutic activity against human benign tumors and papillomas.

To examine if HAMLET kills human cancer cell, we selected to perform a topical study in patients with bladder cancers. Patients were enrolled at the Urology department, Lund University Hospital after written consent. During the week before scheduled surgery the patient received five daily instillations of HAMLET (30ml,25mg/ml) and were encouraged to resist voiding as long as possible. Urine samples were collected before

and after each instillation. The size and shape of the papillary tumors were documented by photographs via cystoscopy, before the first HAMLET instillation and at surgery. Two patients suffered from CIS (*Cancer in situ*) and their tumor status was documented by biopsies (five at each time) taken before and after the instillation period.
We immediately noticed that the patients shed a lot of cells into the urine after each HAMLET instillation. The shed cells were dead, determined by trypan blue exclusion and showed atypical morphology. We suggested that the cells originated from tumor tissue. In parallel there was a reduction in tumor mass. The shedding was not detected when the patient received HLA, NaCl or PBS instead of HAMLET.
Shed tumor cells were collected and TUNEL staining was performed on cytospinned urine samples. In 6 out of 9 patients we detected TUNEL staining in shed cells and in 3 of this 6 patients the majority of the cells were TUNEL positive indicating that HAMLET had triggered DNA damage and apoptosis like cell death *in vivo*. Furthermore, we detected TUNEL staining in remaining tumor in 6/9 patients but not in biopsies taken from healthy areas of the patient bladders. HAMLET instillation of the two *CIS* patients also resulted in a rapid cell shedding and several of the biopsies after the instillation period were negative for tumor cells. Healthy tissues were unaffected by the HAMLET instillations.
We concluded that HAMLET triggers the shedding of cancer cells from solid tumors *in vivo*. This shedding was HAMLET-specific, as we did not see any shedding after control instillations. HAMLET thus appears to have a selective death inducing effect on tumor cells. The results also suggest that HAMLET has a potential as topical therapeutic agent against bladder cancer, although further placebo controlled treatment studies in humans are needed.

### Paper V. HAMLET treatment delays bladder cancer development.

The human bladder cancer study was not a treatment study and the instillations were restricted to one week before scheduled surgery. To examine if the effect of HAMLET is of therapeutic value the MB49 mouse model was used. MB49 bladder tumor cell line was originally developed from a 7,12-dimethylenzanthacene-induced bladder cancer in a male C57BL/6 mouse [79] and were kindly donated by A. Loskog, (Uppsala University,

Sweden). The cells are installed into the bladder of female mice and establish tumor growth within days [80]. The tumor cells were shown to be sensitive to HAMLET *in vitro* and were killed in a dose-dependent manner.

A pre-treatment study was performed to verify the model, and to establish diagnostic tools. Tumor cells were installed after pre-treatment with poly-L-Lysine. Urine was collected every second day for erythrocyte quantification and mice were sacrificed at regular intervals over a 14 days period to quantify tumor development. Animal weight was registered before tumor cell instillation and at sacrifice. The bladders were weighed, fixed in PFA and cryo-sectioned before haematoxylin eosin staining. In most cases, tumors could be separated from healthy tissue, except during the first days. By day 10-14, the tumors filled the bladder lumen. From the pre-treatment study we concluded that the therapeutic time-window was narrow as tumors had to be detectable by microscopy but not jeopardy animal survival.

To evaluate the therapeutic effect of HAMLET in the mouse bladder model, five intravesicle HAMLET or placebo instillations were performed into tumor bearing bladders. The instillation protocol was slightly modified compared to the human study. The best result was obtained when the mice received the three first instillations on daily basis and the two last once every second day. The animals were anesthetized during instillations and it was not considered safe for the animals to be subjected to this procedure daily for the whole treatment period. The day after the last instillation the animals were sacrificed and the bladder isolated, weighed and fixed for histological examination. The tumor area was defined and used as a measure of the tumor growth.

The largest tumor area was compared to the total bladder area and the results showed a reduced tumor growth in animals treated with HAMLET compared to the placebo group ($p=0.02$). Evidence of apoptosis was obtained by TUNEL staining. Within the tumors, areas with DNA breakage were seen after HAMLET treatment but not in healthy bladders treated with HAMLET. By whole body imaging, Alexa-labeled HAMLET was shown to be retained in tumor bearing bladders (4 h or more). Native ALA was not retained by the tumors and it was not possible to detect HAMLET after instillation into a healthy bladder. This results indicating that HAMLET uptake is specific for tumor cells.

We concluded from paper V that the C57BL/6 model is reliable and mimics important aspects of human disease. We confirmed the human study showing that HAMLET has a therapeutic effect by killing tumor cells *in vivo* and delaying tumor growth.

## General discussion

HAMLET was discovered by serendipity. During studies of anti-adhesive molecules in human milk, tumor cells were shown to undergo substantial morphological changes when mixed with casein. The protein of the active casein fraction was identified as alphalactalbumin, a whey protein needed for lactose production but with no known tumoricidal activity. Later, it was shown that alphalactalbumin did not act on its own but in a complex with oleic acid. Native, inactive alphalactalbumin could be converted to HAMLET by incorporation of the fatty acid during a chromatographic step. Bovine alphalactalbumin also forms a complex, BAMLET, with similar activity as HAMLET.

These observations have been reproduced and verified by several groups [81,82,83,84], using a variety of techniques. HAMLET/BAMLET like complexes have either been produced using the original, chromatographic method described by Svensson et al [34] or by mixing of the two components under different conditions. Permyakov et al used de-calcified alphalactalbumin and titrated small aliquots of oleic acid into the protein solution under constant mixing. The experiments were performed at different temperatures and monitored by spectroscopy to determine the critical micellar concentration (CMC) of oleic acid in the protein solution [82]. The formed complex was dialyzed against water before lyophilization. They used HAMLET produced according to the chromatographic method as control. The Fontana group also used two methods to form complexes. By mixing protein at pH 7.4 with 10-15 molar equivalents of oleic acid, active complexes were formed after 1 hour at room temperature, as analyzed in cytotoxic assays directly after incubation.  In parallel, the Fontana group used the chromatographic method, as well. In the mixing protocols, there is no purification step to remove excess free fatty acid, and thus, the cytotoxic activity of the complex *per se* is difficult to evaluate [83].
Zhang et al [85] pointed out parallels between their method to prevent amyloid formation at low pH and the casein precipitation method used to purify MAL [37]. Bovine apo-alphalactalbumin was mixed at low pH with a stock solution of oleic acid and incubated in 37°C for up to 2 hours. The cytotoxicity of the formed complexes was measured and confirmed by trypan blue exclusion and MTT assays. They also used Hoechst33258 to verify nuclear fragmentation and flow cytometry confirmed apoptosis

by PS exposure and hypodiploid DNA peaks. However, they did not use converted material as a control and did not state that their cytotoxic complexes were comparable to HAMLET.

Alphalactalbumin is known to form partially unfolded states after treatments including low pH, chelator, detergents and heat (summarized in table 1). Low pH and EDTA have been shown to trigger the structural changes needed for conversion to HAMLET [34,37]. Kamijima et al recently used heat to partly unfold human or bovine alphalactalbumin before incorporation of oleic acid. The protein was dissolved in PBS at neutral pH and oleic acid was added by mixing. The solutions were either kept at room temperature or heated to 50 or 60°C before excess oleic acid was removed by centrifugation. The resulting complexes were tested for tumoricidal activity and conventionally produced HAMLET was used as control. [84]. In contrast to Fontana the group of Kamijima et al. did not obtain an active complex by mixing alphalactalbumin with oleic acid at room temperature [84]. In contrast, complexes were formed when the protein solution was heated during the incubation. The activity of the heat-formed complexes was comparable to HAMLET prepared by the chromatographic method. This indicates that heat induced structural changes of alphalactalbumin (T-form, table 1) promote the formation of HAMLET like complexes. It is not clear whether the high concentration of oleic acid (1:120 mol equivalents) is required for complex formation and if centrifugation is sufficient to remove the unbound fatty acid that might influence the activity (see below).

Recombinant mutant proteins have also been used for conversion, to address questions about specific domains/properties of the complexes. , The D87A, mutant lacking the capacity for high affinity calcium binding and does not fold to the native state but forms an active, tumoricidal complex showing that bound calcium is not required for HAMLET activity and suggesting that the native state is not involved. This was confirmed using the rHLA$^{All-Ala}$ mutant, lacking disulphide bonds and thus unable to revert from a partially unfolded state to the native state. Near UV CD spectra of the proteins showed a loss of signal confirming the lack of native conformation. The recombinant proteins were tumoricidal after conversion on the oleic acid conditioned matrix without any unfolding pre-treatment. [28,29]. The results indicate that the unfolding method is not

crucial for HAMLET formation. The different methods used such as low pH, chelators, heat and strategic mutations all drive a structural change of alphalactalbumin needed for the incorporation of oleic acid.

We routinely use oleic acid in HAMLET production since it is the most abundant fatty acid in human milk and a part of the natural complex. The second step in HAMLET formation is the incorporation of oleic acid. Several fatty acids, differing in both carbon chain length and grade of saturation [25] have been shown to form complexes with alphalactalbumin but only complexes with oleic acid and vaccenic acid (both 18:1 fatty acid) kill tumor cells efficiently. This specificity, might be due as much to the cellular recognition of these fatty acids as to the protein:lipid interaction and needs further study.

Using the chromatographic method, fatty acids bound to the protein are eluted at high salt and unbound fatty acids are washed off the column before the protein is added. In the mixing experiments it is not always clear if unbound fatty acid is removed or present in the different cellular assays [84,86]. The protein/fatty acid stoichiometry is therefore not finally determined [82]. Mixing experiments, performed by Knyazeva et al [82] showed that the complexes formed at different temperature differed in protein-lipid ratios (1: 2.9 at 17 °C and 1: 9 at 45°C) but they did not identify any activity differences between the complexes. HAMLET, converted as described by Svensson et al [34] showed slightly lower $IC_{50}$ values in their experiments.

Oleic acid is not toxic to tumor cells at concentrations found in HAMLET. Bound to albumin, oleic acid, as well as other fatty acids, oleic acid is constantly used as energy and building blocks for prostaglandins and membrane phospholipis [87,88]. Initial experiments with oleic acid saturated serum albumin (manuscript in preparation) show that despite higher fatty acid uptake than via HAMLET the cells do not die when exposed to the oleic acid-albumin complex. The two proteins may thus deliver the fatty acids in ways that activate different signaling events.

The protein-lipid ratio has been determined in several HAMLET batches produced in Lund over the years. By acid hydrolysis and GC/MS, the ratio has been determined to be

1:4-8 before as well as after gelfiltration (PD10). This indicates that while the preparations does not contain unbound fatty acids, small differences in the fatty acid content are difficult to avoid, and so far, such differences have not been possible to link to the activity.

It is clear from the data described above that cytotoxic complexes between alphalactalbumin and oleic acid are formed under several different conditions. It is also obvious that the cellular data from the different groups are hard to interpret due to the differences in complex composition. Several groups are claiming that a mixing method is superior to the chromatographic method described by Svensson et al [34]. We would like to argue the opposite. The chromatography method has proven to be highly reproducible over the years and the batch-variations are small. The conversion yield can constantly be monitored and the HAMLET fraction are well separated from un-converted protein as well as un-bound fatty acid. The method can be scaled up (see section HAMLET production) and has, successfully, been used for GMP production. It is interesting to notice that the differences in lipid content of the formed complexes is, if not neglected, at least regarded as of minor interest by the different groups. In cell biology the effects of lipids on homeostasis are extremely important and we predict that the fatty acid stoichiometry will prove essential to reproduce and compare cellular responses to different HAMLET or HAMLET like preparations.

As the major structural change in alphalactalmin fold appears to involve the beta-sheet region [1] it is tempting to suggest that the fatty acid binding site may be located in this domain or at surfaces in the cleft between the two domains, which are exposed when the β-sheets are less ordered. The contribution of the amino acids in the calcium site to fatty acid binding was excluded, as point mutation in the calcium-binding site did not change fatty acid binding or tumoricidal activity (L. Berliner and C. Brookes, un-published data). In studies by of Casbarra et al Hydrogen/Deuterium (H/D) exchange and limited proteolysis was used to study structural differences between HAMLET, native- and apo-alphalactalbumin [89]. They show that alphalactalbumin in HAMLET was highly available for hydrogen-exchange and incorporated even more deuterium atoms than apo-alphalactalbumin. Furthermore, HAMLET was sensitive to proteolysis but only at a few, very specific, sites within the β-sheet domain. The results also indicate that the

binding of fatty acid in the HAMLET complex might reinforce unfolding leading to an even more unstructured β-sheet domain. Tolin et al suggest that there is no single fatty acid binding site in HAMLET [86]. They show that fatty acids can be incorporated by several alphalactalbumin peptides separated by reversed phase high-performance liquid chromatography after limited proteolysis, converted on oleic acid conditioned matrix to form tumoricidal active complexes. It is unclear if these active fragments reflect the properties of the intact HAMLET complex or represent a different mode of action. Tolin et al. also suggest that the protein acts as a carrier and that the fatty acid is the major active component.

The ELOA-complex formed from Equine (horse) lysozyme, binds oleic acid under similar conditions as HAMLET and displays tumoricidal activity. Lysozyme is a close structural homologue of alphalactalbumin. In contrast to hen-egg white lysozyme the equine (horse) lysozyme contains a calcium-binding site, like alphalactalbumin. The equine protein is therefore regarded as a structural intermediate between the two related proteins. Wilhelm et al speculate that equine lysozyme binds oleic acid at several different positions in the protein [90]. Although the protein has a calcium site, the protein does not require unfolding to form a complex with oleic acid on the chromatography matrix. In contact with the fatty acid, the lysozyme protein is thought to gradually unfold and expose new binding sites for oleic acid. The formed complexes were shown by NMR to vary in protein lipid ratio from 1:9 to 1:48 but the activity was correlate to protein concentration rather than lipid content [90]. Hen-egg lysozyme has been subjected to conversion experiments on oleic acid conditioned matrix. The protein was not retained on the DEAE Trisacryl M matrix and did not produce any active complex either with or without EDTA pretreatment (data not published).

The major structural question to be solved is how to define “HAMLET”. As presented here, cytotoxic complexes between oleic acid and alphalactalbum as well as lysozyme can be formed in several ways. It is unclear if different methods result in the formation of the same molecule, however, and if the cell death mechanisms differ. Will the cellular responses may be similar, irrespective of fatty acid content, protein or peptides carrying oleic acid and the ability of the protein to revert to the native state. On-going 2D-NMR studies as well as crystallization experiments aim to reveal the structure of HAMLET, as

originally describes, as well as differences between native alphalactalbumin and HAMLET. Different strategies, based on recombinant proteins and peptides, are also used to determine the minimal protein structures needed for fatty acid binding or/and activity.

HAMLET activates several cell death mechanisms and we have proposed the “hydra concept” to illustrate the multi-faceted cellular response [91]. The broad tumoricidal spectrum of HAMLET indicates that several signaling pathways are affected. HAMLET has been shown to target several organelles and molecules in tumor cells. Düringer et al showed in several ways that HAMLET bound strongly to isolated histone proteins and that HAMLET co-localizes with GFP-tagged histones in the nuclei of tumor cells treated with HAMLET. Permyakov and co-authors pointed out that alphalactalbumin does not have to be converted to HAMLET to bind to histones *in vitro* and suggested that the interaction is based on electrostatic interaction. Furthermore, they acknowledged, however, that native alphalactalbumin would not reach the nuclei of intact tumor cells, as HAMLET but not the native protein is translocated to the nuclei in living tumor cells.

Membrane interactions with small unilamellar DPPC (dipalmittoylohosphatidyl choline) vesicles were recently described by Zherelova [92]. Vesicles were prepared according to Berliner et al [93] and mixed with the different protein and protein complexes for 15 hours at 4°C. Unbound proteins were separated from the vesicles by gel filtration. They show that the OA-containing complexes bound stronger to vesicles than the native and apo-proteins. Those results are in line with what was observed in the binding experiments presented in paper III. In contrast to Zherelova et al, the interaction between HAMLET and liposomes were measured at room temperature or at 37°C. By fluorescence microscopy, leakage experiments and SPR the binding was shown to be fast and differences between HAMLET and alphalactalbumin were detected in less than one hour. LA-OA-17 was also shown to affect the transmembrane currents by modifying the plasmalemmal ionic channels of *Chara coralina* cells. The effect on current and nonspecific $K^+$ leakage was not as pronounced when HAMLET, prepared by the chromatography method, was used. In our experiment we show leakage of small molecules through the membranes after incubation with HAMLET but either Zherelova or our work showed uptake of HAMLET/LA-OA-17 into the vesicles.

As summarized previously (page 28), HAMLET-treated cells show signs of apoptosis, autophagy, proteasome overload and more recently ER stress and oncogene related activity have been identified [15,54,65,73]. Recently, Rammer et al [81] showed that BAMLET induces lysosomal leakage of cathepsin leading to activation of programmed apoptosis-like cell death via Bax [81]. They were also able to block some of the BAMLET induced cell death by over-expression of lysosomal stabilizing proteins. This is yet another activated pathway, leading to cell death. In their paper they used a whole range of different tumor cells and verified the activity of their BAMLET preparation in comparison with HAMLET. The major part of the study is performed in MCF-7 breast cancer cells. While HAMLET kills tumor cells broadly, it is possible that each cell type will respond with the activation of specific signaling pathways according to its genetic profile and that the pathways will converge on a few shared death mechanisms. Thus, different cell types may provide important pieces in the puzzle that is HAMLET's mode of action.

## Short summery of the included papers

### Paper I

MAL, purified from human milk casein, kills tumor cells and contains the whey protein alphalactalbumin in a partially unfolding state. Native alphalactalbumin does not kill tumor cells and the activity was shown to depend on the binding of a cofactor. The cofactor was subsequently identified as oleic acid. Purified native HLA, can deliberately, be converted to a complex inducing cell death. By the removal of the calcium ion the protein structure is slightly changed. When exposed to oleic acid in a chromatographic step the complex is formed and eluted in high salt buffer. The complex was named HAMLET (**H**uman **a**lphalactalbumin **m**ade **le**thal to **t**umor cells).

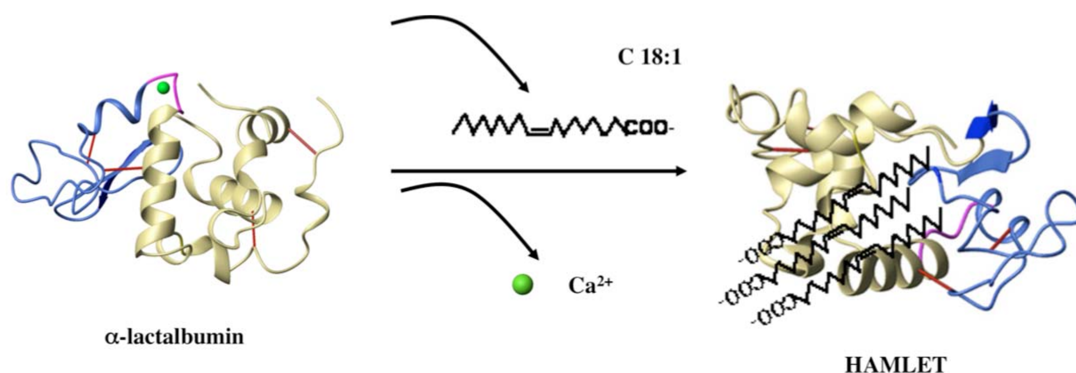



**Figure.** Schematic figure of the HAMLET complex formation. Native alphalactalbumin is subjected to EDTA to remove the calcium ion. The EDTA treated protein is subjected to C18:1 conditioned ion-exchange matrix and has, opon elution, incorporated the fatty acid into the protein structure.

### Paper II

The alphalactalbumin sequences have been conserved during evolution. The bovine protein can be converted to BAMLET resembling HAMLET in structure and activity. We showed that casein from different species do not contain HAMLET like complexes as found in the human. However, active complexes were obtained when purified alphalactalbumin from the different species was converted on oleic acid conditioned matrixes. The results suggest that the natural occurring differences in the amino acid sequences do not prevent the HAMLET formation but the oleic acid in the animal casein is not accessible to complex formation.

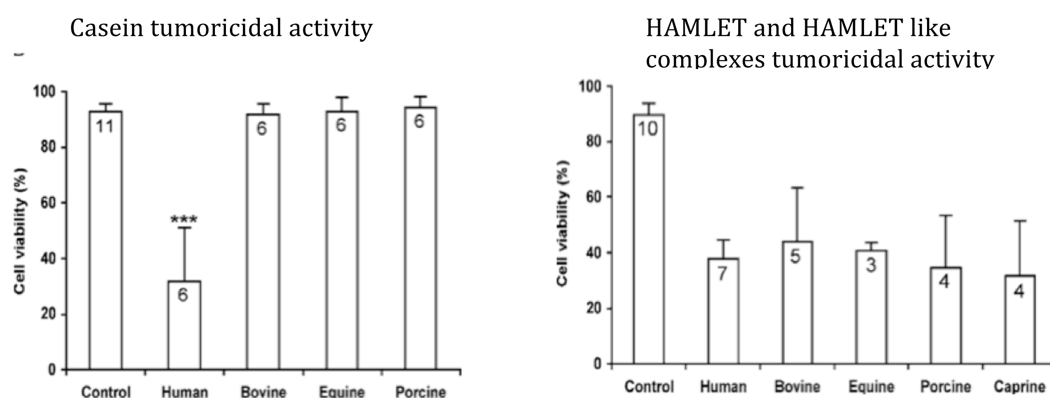



**Figure:** Cell viability measured after incubation with casein (B) isolated from different animals an HAMLET like complexes converted from purified alphalactalbumin and oleic acid. The tumor cell killing activity is only seen in casein made from human milk where else all of the converted complexes show similar activity as HAMLET.

**Paper III**

In paper III we investigate if the previously described interaction between non-native forms of HLA and artificial membranes can facilitate the binding of HAMLET to cell surface. We show that HAMLET interacts with artificial- as well as and tumor cell derived membranes and the interaction disturbs the membrane morphology and cause vesicle leakage. There was no evidence of HAMLET uptake into the vesicles as showed below by confocal microscopy.

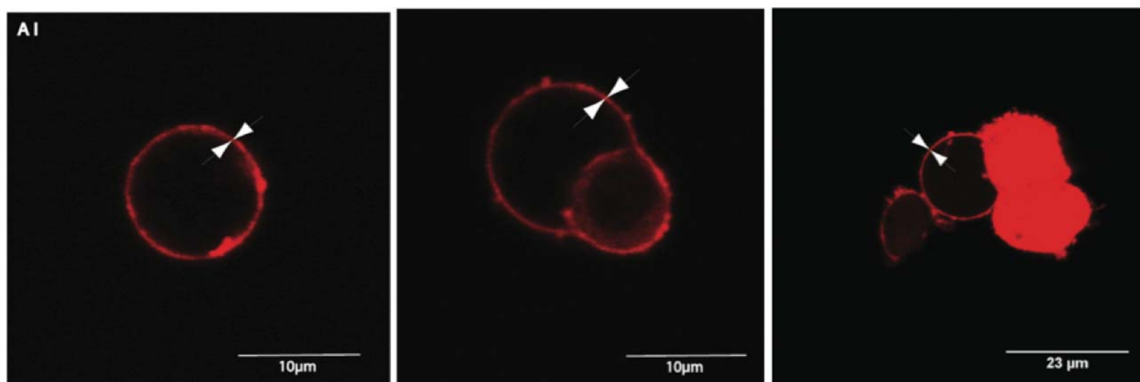



**Figure**. Confocal imagines showing Alexa-HAMLET (red) binding to plasma membrane besicles (PMV). .

## Paper IV

During the week before scheduled surgery, seven patients with papillary tumors and two patients with CIS were instilled with HAMLET solution once a day. In the urine sample collected after HAMLET instillations, large numbers of dead cells were detected. The morphology of the shedded cells indicated that they originated from the tumors and a macroscopically detectable reduction in tumor volume was also seen. Using tissue sections, we showed that HAMLET instillations caused cell death in the tumor cells *in vivo* but we could not detect any damage to surrounding healthy tissue.

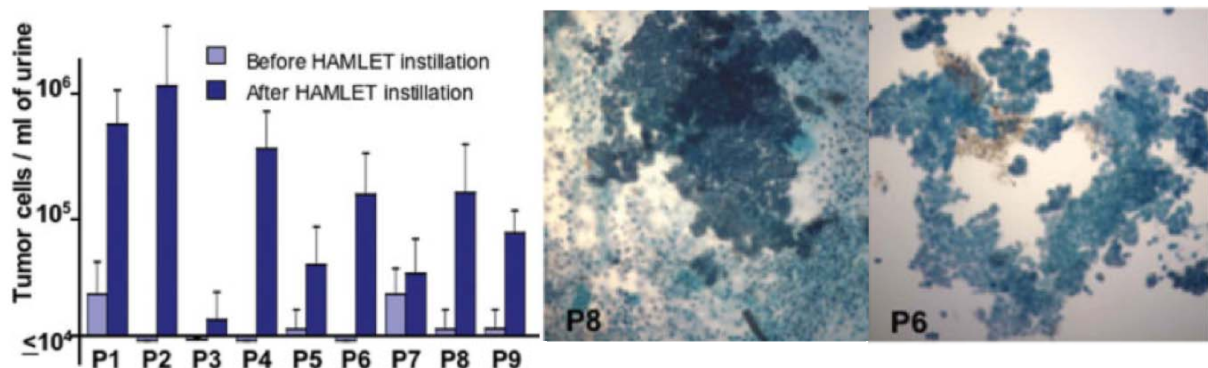



**Figure:** Diagram is showing the mean of shedded cells found in the urine before (light blue) and after (dark blue) the HAMLET instillations. To the right two pictures showing examples of aggregates of dead (trypan blue) cells found in the urine after HAMLET instillations

## Paper V

A bladder cancer mouse model was used to examine if HAMLET is therapeutic *in vivo*. MB49 tumors were established by injection of tumor cells into the bladder of mice pre-treated with poly-L-lysine and rapidly growing tumors can be detected within a week. Five intravesicle instillations with HAMLET or control solution were given before the mice were sacrificed and the bladder was isolated and preserved for sectioning. We show that the HAMLET instillation caused a delay in tumor progression and there were more tumor free animals among the HAMLET treated, compare to the control group.

## Acknowledgements

I have imagined writing this part of my thesis several times. Now, when it finally is time, it scares me! Why? For the obvious risk to forget someone! So, initially, I would like to say to those I might leave out….

THANK YOU!

I start by thanking my professor and supervisor, **Catharina Svanborg.** You are obviously, the reason for me writing this book at all! Taking me "on-board", so many years ago, allowed me to take part of the HAMLET discovery. You have also shown me that enthusiasm and courage is essential parts of research as well as in life. I thank you for the first inspiring 20 years and I look forward for the ones to come.

Research is partially very frustrating and would be even more so if you did not share your worries and sorrows with a bunch of great lab-mates. Several HAMLET group members are already graduated and have left the group physically but are still present within the lab due to their work and personalities.

Special thanks to **Anders Håkansson** and **Malin Svensson**, the first PhD students in the project and with whom I shared the first chromatograms of conversion. I enjoyed those days and I appreciate you still keep in touch. Anders, please, can finally admit that you enjoyed "Cats"? And Malin, I hope you dress-nightmares are all gone!

Special thanks also to **Jenny Pettersson-Kastberg** who understands my interest in the HAMLET molecule and with whom I share unforgettable memories such as: pig-milking expeditions, hotel confusions in Washington and problems to convince the purchaser in Hörby. You are great!

Previous HAMLET members also includes **Hemant Sabharwal, Anita Saraf, Christina Hansson, Lotta Gustafsson, Oskar Hallgren, Caroline Düringer, Helena Ågerstam, Patric Brest, Chimene Morheillon, Camilla Svensson, Irene Lejonhufvud** and **Charlotte Böjers** who all contributed substantially to the work included in this thesis and a major reason for me to enjoy my work.
The current members keeps up the good spirit and includes **Maria Trulsson** who, beside work issues also encourage my struggling with horses, **Sonja Aits** who bakes marvelous apple cakes and is a master of western blot **Petter Storm,** reliable in every way BUT addicted to Kalmar FF and **Susan Basiri** who keep the HAMLET production up and running, sharing (?) my passion for chromatograms.
I would also like to thank **Ken Mok** at Dublin University, a key member of the HAMLET group with an interesting interpretation of Swedish traditional songs. Among all our lovely co-workers at Singapore Immunology Network (SIgN), I especially would like to mention **Pricsilla Lim Lay Ken** for taken such good care of both me and HAMLET in Singapore.

Several other persons are included as co-authors in the papers, indicating fruitful collaborations with other groups and laboratories. I would like to thank them here as well.
**Sara Linse**, at the department of Biochemistry, whose enthusiasm and knowledge contributed substantially to the structural work of HAMLET.

**Björn Wullt**, medical doctor and supervising the clinical study. Together with **Wiking Månsson** and **Eva Ljunggren** at the urology department he made it possible to perform the human bladder cancer study.

I would also like to thank **Majlis Svensson**, my collogue and dear friend in the UTI group, who knows everything there is to know about laboratory work. She, as well as **Yochuan Hou** and **Bo Holmqvist** were part of the *in vivo* study of paper V.

Paper III is based on collaboration with **Öjvind Halskau** och **Aurora Martinez** at the university of Bergen, Norway and **Roger Karlsson, Ingela Lanekov** and **Maja Purchades** at the University of Gothenburg. It has been great fun and inspiring to visit your labs, learning new techniques and to enjoy sushi!

Sharing everything from pipettes to laughs the colleagues in the UTI group is part of the inspiring environment of the lab. **Bryndis, Jenny, Hans, Manisha** and **Natalya** are present members but several others have previously contributed to the group. Thanks to you as well!

**Gittan, Gertrud** and **Eva,** colleagues as well as friends, I would like to mention It is comforting to have you around, showing genuine interest in persons you care about. Thank you for your support during so many years!

Understanding and supportive friends and relatives are important to me. I like to mention the "**P2-gänget+respektive**" with whom I share a lot of fun and unforgettable moments at the side of the soccer field. A special thanks to **Susanne Gunnarsson**, a dear friend who have helped me numbers of times to keep the every-day-life rolling.
The **"Midsommar/Nyårs-gänget**", "**Sellbergs**", "**Nordlunds**" och **Anna PH** is a bunch of lovely people who knows how to enjoy life, several with a growing interest in golf.
**Lena, Helen** and **Rozita**, the "girls" with whom I once graduated with and still meet on regular basis. Nowadays we rather go shopping than nurse the "labass"-issues**,** once so important. **"My mothers"** actually referees to two groups of people I would like to thank. The first being the milk donators and to whom I become quite close to during their lactating period. The other group being **Eva, Eva** and **Louise** with whom I shared the first experience of becoming a mother and still meet at obscure pubs whenever we find the time. Thank you all for being my "supportive matrix"!

Finally I would like to express my gratitude to my relatives and family. My mother, **Kerstin**, and sister, **Liselotte**, are important part of my world. Neither interested in protein purification but always encouraging and supporting me in every possible way! Im lucky to have the two of you and hope you feel that you are sharing this work with me. **Andreas, Sara** and **Rebecca** supportive and inspiring to have you around! I like to mention **Gunnel**, my mother in law, and important part of the family as well.

My husband **Ulf** and our children, **Camilla** and **Sebastian,** are the ones I owe the most. Although, HAMLET has been a substantial part of our lives, I never experienced any "jealously". I am so grateful for your love and support, showed in a thousand ways, over the years!

//Anki

This thesis were supported by grants from:
Swedish Cancer Society, , The Lund Family Grant from the American Cancer Society, Swedish Medical Research Council, Swedish Natural Science Research Council, Swedish Pediatric Cancer Society, The Segerfalk Foundation, The Österlund Foundation, The Lund Hospital Foundation, Royal Physiographic Society, Anna-Lisa, Sven-Erik Lundgren Foundation, Knut and Alice Wallenberg foundation, Inga-Britt and Arne Lundbergs Foundation and the John and Augusta Person Foundation for Medical Research.

I would also like to thank the patients participating in the clinical study of paper IV.

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