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Several aspects of pathogenesis of *Trichomonas vaginalis*

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Acknowledgments

Contents

Abstract

The pathobiology of *Trichomonas vaginalis* involves direct and indirect interactions with host tissue, bacteria and viruses. In this work, we investigated adhesion and interaction with human microbiota, pathogenic mechanisms of protozoan.
We characterized a new protein, TVAG339720, belonging to M60-like/PF13402 domain-containing proteins. M60-like domains are shared by proteins from several mucosal microbes, hypothetically relating with epithelial cells. TVAG339720 is characterized by a signal peptide, a transmembran domain, and putative carbohydrate binding modules, PA14 and GBDL, supposed to bind to heparin and heparan sulphate (HS). HS are sugars forming proteoglycans, a component of epithelial cells glycocalyx. We tested protease activity of TVAG339720 towards proteoglycans and interaction between CBMs, heparin and HS. Although the target of TVAG339720-M60L is still unknown, the bounds between TVAG339720-CBMs and HS suggest that these proteases play a role in adhesion to epithelial layer.
T.vaginalis interactions with microbes of urogenital tract represent another pathogenic mechanism. We focused on symbiosis established with *Mycoplasma hominis*, studying how *M.hominis* influences *T.vaginalis* pathobiology *in vitro*. Comparing the ATP produced by free-protozoan and *T.vaginalis* with *M.hominis* and evaluating mycoplasma ability to influence nitric oxide production by macrophage in *T.vaginalis* infections, we can assert that this is a mutually beneficial endosymbiotic relationship.

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Abbreviations

A280	nm Absorbance at a wavelength of 280 nm
ADH	Arginine dihydrolase pathway
ADI	Arginine deiminase
AGE	Agarose gel electrophoresis
BACON	Bacteroidetes-Associated Carbohydrate-binding often N-terminal CBM
BLAST	Basic Local Alignment Search Tool
BspA	Bacteroides surface protein A
CAZy	Carbohydrate active enzymes database
CBM	Carbohydrate-binding module
CFE	Cell-free extract
CK	Carbamate kinase
cOCT	Catabolic ornithine carbamyltransferase
CS	Chondroitin sulfate
DNA	Deoxyribonucleic acid
EC	Enzyme commission number
ECM	Extracellular matrix proteins
EDTA	Ethylenediaminetetraacetic acid

FT	Flow-through sample during IMAC
GBDL	Galactose-binding domain-like
GiardiaDB	*Giardia lamblia* genomic databse
GlcA	d-Glucuronic acid
GlcN	D-glucosamine
GP63	Major membrane glycoprotein Leishmania spp
GR	Glucose-restriction
His6-tag	Polyhistidine tag
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IdoA	l-Iduronic acid
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITC	Isothermal titration calorimetry
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOD	Thermococcus kodakaraensis DNA polymerase enzyme
LPG	lipophosphoglycan
LTG	Lateral gene transfer
MEROPS	On-line database for peptidases
Mh	Mycoplasma hominis
MMPs	Matrix metalloproteinases
MT1-MMPs	membrane type MMPs
NAD+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NC	Nitrocellulose membrane
NO	Nitric oxide
OD600nm	Optical Density at 600nm
PA14	CBM named after the anthrax Protective Antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFAM	On-line database of protein families
SAGE	SDS-agarose gel electrophoresis
SDS	Sodium dodecyl sulfate

SDS-PAGE	SDS polyacrylamide gel electrophoresis
STD	Sexually transmitted diseases
Syd-1	Syndecan-1
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TrichDB	*Trichomonas vaginalis* database
Tv	Trichomonas vaginalis G3
TvBspA	BspA-like proteins in *T.vaginalis*
Tv-MhMPM02	*T.vaginalis* G3 in symbiosis with *M.hominis* MPM02
TVV	*T.vaginalis* virus
VEC	Vaginal epithelial cell

CHAPTER I

General Introduction

I.1 Introduction

Sexually transmitted diseases (STD) are a major global health problem. Each year, an estimated 500 million people acquire one of four sexually transmitted infections: chlamydia, gonorrhoea, syphilis and trichomoniasis. Moreover, more than 530 million people are living with HSV2 and more than 290 million women have an HPV infection [1].

Trichomoniasis is the most common non-viral, curable, STD worldwide that annually affects millions of people[1]. The causative agent of infection is *Trichomonas vaginalis*, obligate extracellular mucosal parasite that induce significant health sequelae in both men and women. In women, symptoms range from a silent form to important complications including pelvic inflammatory disease [2], invasive cervical cancer [3], sterility, pregnancy and postpartum problems [4, 5]. In men, the infection occurs mainly without symptoms, complicating its diagnosis and control. Recently, trichomoniasis has been associated with aggressive prostate cancers [6-8]. Moreover, *T.vaginalis* infection is epidemiologically associated with HIV [9-11]. Despite the high prevalence of trichomoniasis and the complication associated with the disease, little is known about parasite or host factors involved in pathogenesis [12, 13].

I.2 *Trichomonas vaginalis*

Trichomonas vaginalis is an anaerobic flagellated eukaryotic protozoan that infects the urogenital tract of human body. This pathogen varies in size and shape; with the average length and width being 10 and 7 μm respectively [2]. The two best characterized forms are the trophozoite and the ameboid. The trophozoite is the

infective form, motile stage of protozoa. In this stage *T.vaginalis* tends to be uniform, i.e. pyriform or pear-like cell. Upon contact *in vivo* with epithelial cells from the vagina, cervix, urethra, prostate and extracellular matrix (ECM) proteins, the protozoan can rapidly switch from trophozoite to ameboid form, a pancake shape that allows an increasing of the surface contact [2, 14]. In a process called swarming, trophozoites are able to attach one to another by their pseudopods forming aggregates consisting of numerous cells. The exact role of these aggregates is not yet clear. Pereira-Naves et al have shown the presence of third form of *T.vaginalis,* which can be induced *in vitro* upon exposure of trophozoites to cold and drugs [15]. Even for this shape, called pseudocyst, the significance during the infection is unknown [16]. The organism possesses four anterior flagella and a fifth one comes along with its undulating membrane, a large nucleus, and an axostyle, which bisects the protozoan longitudinally. Moreover, *T.vaginalis* cytosol is glycogen rich and contains other internal organelles such a cytoskeleton, costa, pelta and hydrogenosomes (Figure I.1). The hydrogenosomes are double membrane organelles that share common ancestry with mitochondria, [17, 18], and are involved in production of ATP, acetate, carbon dioxide and hydrogen as end products from pyruvate and malate substrates [19]. *T.vaginalis* lacks conventional mitochondria and peroxisomes but contains hydrogenosome, which are a typical feature of parabasalid lineage to which *T.vaginalis* belongs (Table I.1). Hydrogenosomes are also found in several anaerobic eukaryotic microbes [20].

T.vaginalis is an unusual eukaryote showing remarkably similarity to primitive anaerobic bacteria, in terms of its carbohydrate and energy metabolism. In fact, *T.vaginalis* mainly obtains energy from fermentative carbohydrate metabolism under both anaerobic and aerobic conditions [19]. Glucose is converted to pyruvate in cytosol and subsequently metabolized in the hydrogenosome by fermentative oxidation. Hydrogenosome produces ATP by substrate-level phosphorilation [21]. Trichomonads require unusually high concentrations of iron in *in vitro* cultures, likely for the dependence of *T.vaginalis* upon the activities of heme or iron-sulfur (FeS) cluster-containing proteins. FeS are complex prosthetic groups that bind iron and mediate vital energy-conserving reactions in the parasite's hydrogenosomes [22]. High extracellular level of iron may also be

required from trichomonads to furnish the turnover of FeS proteins, given that this pathogen apparently lacks substantial levels of iron-storage proteins, such as ferritin [23].Several studies have investigated the effect of iron limitation on *T. vaginalis* morphology and overall proteome change, showing how cells from iron-depleted medium displayed altered morphology, including the internalization of flagella and the axostyle and transformation to a larger and rounded shape [24, 25]. Moreover, iron deficiency led to the upregulation of proteins involved in iron-sulfur cluster assembly and the downregulation of enzymes involved in carbohydrate metabolism [26].

Furthermore, the pathogen lacks the ability to synthesize many macromolecules *de novo*, particularly purines, pyrimidines, and many lipids, and acquires these nutrients form vaginal secretions or though phagocytosis of bacteria, vaginal epithelial cells (VECs) and erythrocytes [27-29].

Table I.1 - Classification of *T.vaginalis*.

Domain:	Eukarya
Kingdom:	Protista
Phylum:	Metamonada
Class:	Parabasalia
Order	Trichomonadida
Family:	Trichomonadidae
Genus:	Trichomonas
Species:	*Trichomonas vaginalis*

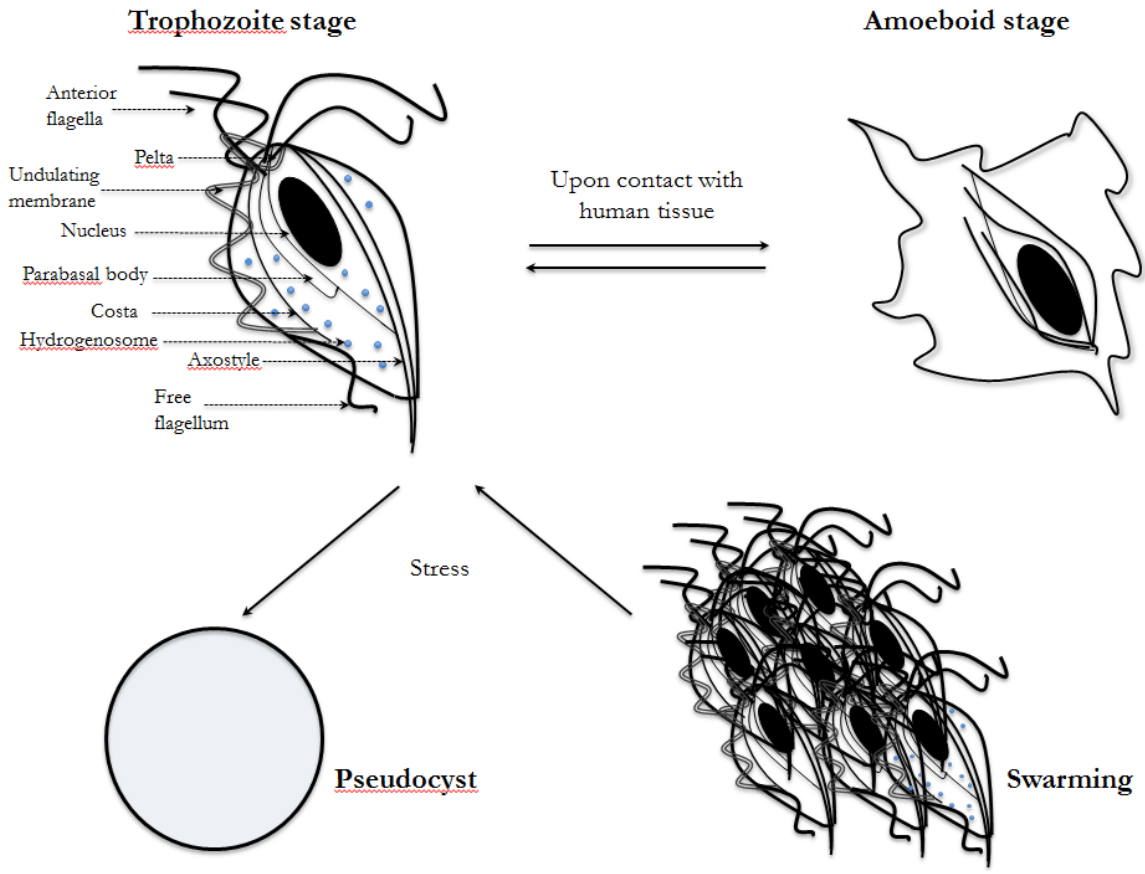


Figure I.1 - Various cellular forms of *T.vaginalis*.

Trophozoite stage shows the morphological features.

I.3 *Trichomonas vaginalis* pathogenesis

T.vaginalis is transmitted from person to person through sexually intercourse [14]. The life cycle consists of two stages, the infective and diagnostic stages. The trophozoites attach to mucosal surfaces of urogenital tract and divides by longitudinal binary fission.

Successful colonization of the host mucosa by *T.vaginalis* is the result of multiple pathogenic mechanisms, including adhesion; secretion of cytotoxic molecules and soluble factors; interaction with member of vaginal microbiome; evasion of host immune system and regulation the development of the immune response [14, 30].

I.4 Adhesion

As mucosal microbial pathogens, *T.vaginalis* must adhere to epithelial cells as an initial step towards colonizing the host and establishing infections [30]. *T.vaginalis* adherence to host cell is mediated, in part, by a major lipid-anchored phosphosaccharide, known as lipophosphoglycan (LPG).
LPG is the most abundant component of *T.vaginalis* surface glycocalyx and the alteration of its sugar content reduces both the ability of adhesion and cytotoxicity of protozoan to host cells [31, 32]. *T.vaginalis* LPG binds to mammalian protein galectin-1, in a carbohydrate-dependent manner [33]. Galectin-1 is the only identified human receptor for *T.vaginalis* so far [33].
Moreover, recent compositional and structural analysis of pathogen revealed that LPG has specific domains with proinflammatory properties, modulating inflammatory responses of epithelial cells and macrophages [34].
Although the binding between LPG and galectin-1 may be central in establishing infections, the parasite involves other adhesion factors to establish host-parasite interaction. In fact, surface proteins are expected to be important for initialing and sustaining infections, allowing the parasite to interact with its environment including human epithelial cells, immunocytes and extracellular proteins of the urogenital tract [30].

I.4.1 *Trichomonas vaginalis* surface proteins

In 2007, the entire genome sequence for the *T.vaginalis* strain G3 has been sequenced [35], increasing the interest about molecular and cellular mechanisms of *T.vaginalis*. The genome has ~160 megabases and contains ~60,000 protein coding genes organized into six chromosomes, according to data from the GiardiaDB and TrichDB databases [14, 35]. *T.vaginalis* genome encodes membrane trafficking machinery, pathogenic proteins for endocytosis of host proteins and phagocytosis of bacteria and host cell [35, 36]. Moreover, a large repertoire of genes consistent with carbohydrate and amino acid metabolism, defense against oxidative stress, transport and pathogenesis are contained into genome [30, 35, 37, 38]. A total of 3000 candidate genes for surface molecules

mediating interaction with host tissue, membrane trafficking and signaling have been identified from the sequenced genome and have been divided in ten different protein families [30, 35]. The genome analysis has also indicated that the pathogen is the first eukaryote that does not produce glycosylphosphatidylinositil (GPI) anchor [35]. De Miguel et al, using a proteomic analysis according to BLAST analysis and genome annotation, have identified 411 surface proteins examining six strains of *T.vaginalis*, showing that 63% of these proteins possess a predicted transmembrane domain and/or signal peptide sequence and 35% of total proteins are membrane proteins predicted to have a possible role in *Trichomonas* pathogenesis [30, 35] (Figure I.2).

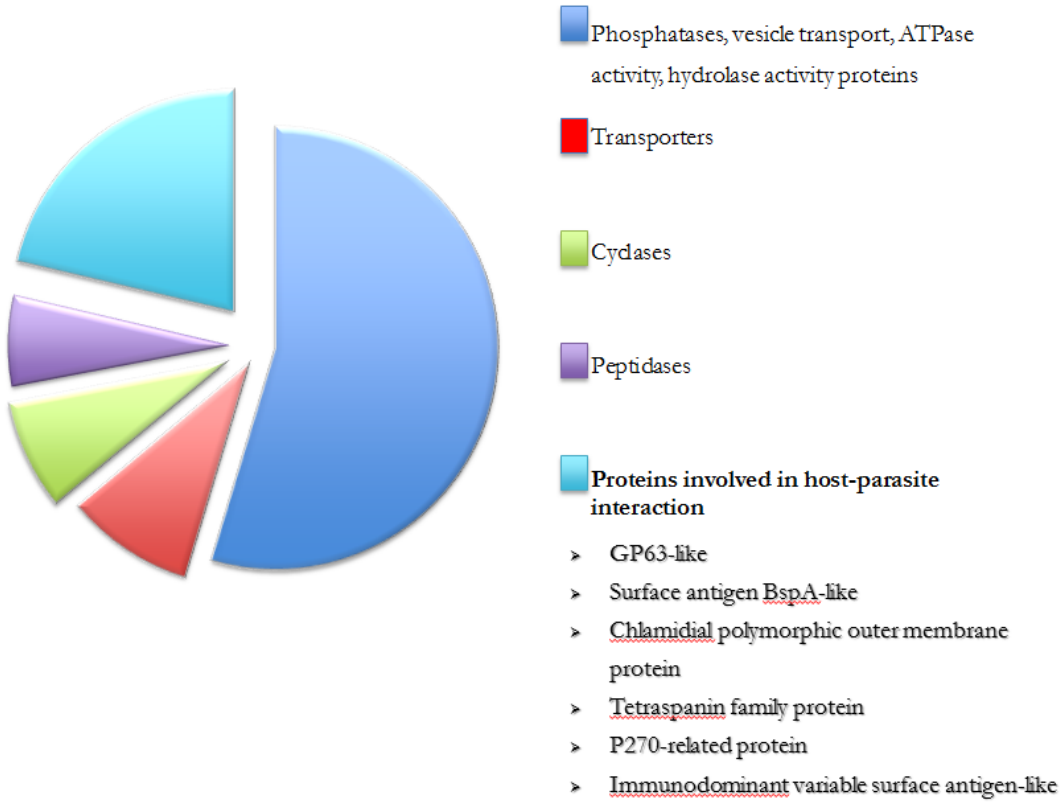


Figure I.2 - Hypothetical membrane proteins of *T.vaginalis*.

Predicted and identified membrane proteins sorted into relative prevalence of functional groups according to proteomic studies, BLAST analysis and genome annotation. (Images modified from de Miguel et al, 2010). Among the 116 hypothetical proteins predicted to possess TMD or SP, ~60% are phosphatase and proteins involved in vesicle transport,

ATPase activity or hydrolase activity; ~8% are transporters. ~8% are cyclases; ~5% are peptidases and ~18% are proteins predicted to be involved in host-parasite interaction. The function of each predicted protein was given through identification of domain that allows the assignment of a predicted function.

BspA-like, GP63-like proteins and adhesins are the major categories of surface proteins and have already been biochemically characterized [14, 30].

Four adhesins, AP65, AP51, AP33 and AP23, are thought to mediate the adhesion of the parasite to the epithelial cells, even if their role as adhesive molecules is highly debatable. These proteins are alternatively expressed on the surface with a highly immunogenic glycoprotein, P270 [2].

TvBspA are the largest gene family encoding potential surface proteins. These proteins share a specific type of leucin-rich repeats (LRRs) named TpLRR (Tp is for *Treponema pallidum*) and they are also identified into *Entamoeba histolytica* and *E.dispar* genomes [12]. LRR-containing proteins were shown to mediate binding to host epithelial cells and/or ECM proteins and were also implicate in bacterial aggregations [39].

The second largest gene family of *T.vaginalis* candidate surface proteins encodes GP63-like proteins. GP63 are metalloproteinase belonging to the metzincin class characterized by the motif HExxHxxGxxH, where x represents any amino acid residues. TvGP63 proteases contain the minimal motif HExxH for zincins and their protease activity is inhibited by the cysteine proteinase inhibitor [40]. GP63 proteases likely play a vital role in *T. vaginalis* infection process, by degrading and binding to various host components.

Another class of surface proteinases is cysteine proteinase, identified by two dimensional (2-D) substrate gel and electrophoresis. CP30 cysteine surface proteinase is a protein of 30kDa involved in binding to HeLa cervical carcinoma cell lines, degrading some ECM proteins and hemoglobin. CP30 is also immunogenic and is secreted into vagina during infection [37].

I.4.1.1 A novel type of surface gluzincin metallopeptidases

Comparative genome studies among bacterial and eukaryotic mucosal microbes, including mutualists and pathogens of invertebrates and vertebrates, have identified several genes and gene families encoding candidate surface proteins and putative enzymes shared through lateral gene transfer (LTG) [30, 41].
One family of *T.vaginalis* candidate surface proteins was annotated as zinc (Zn)-metallopeptidase sharing a novel protein domain termed "M60-like (PF13402) domains"[41]. In profile-profile comparisons, this new domain shows similarity to an existing protein family termed M60-enhancin [MEROPS database][12], characterized by the insect baculovirus (*Lymantria dispar nucleopolyhedrovirus)* and known to be capable of degrading insect intestinal mucins [41].
In M60-like domain is presented HExxH motif or consensus sequence, typical of zinc metalloproteases belonging to the zincin superfamily or clan [42]. The two histidine residues within consensus sequence are ligands of zinc ion (Zn^{2+}), while the glutamic acid (E) residue acts as the catalytic active amino acid [43]. An additional conserved glutamate is presented downstream of the HExxH motif, defining pattern HExxHxE. This motif characterizes the gluzincin-like family of Zn-metallopeptidases and the second E functions as a third zinc binding ligand [41].
InterProScan and Pfam analyses have identified additional domains presents in M60-like containing proteins, the carbohydrate binding modules (CBM). CBMs are involved to binding with specific carbohydrate components within a target substrate and are classified into families on sequence similarities, as defined in the Carbohydrate active enzymes (CAZy)database [41]. CBM families are identified by a number written together with CMB abbreviation, e.g. CBM32 to specify a family with 32 carbohydrate binding module. BACON and PA14 are also carbohydrate binding domains only available from PFAM database and are thought to be involved in glycan binding. Proteins with CBM32 were predominantly associated with vertebrate mucosal surfaces microbes. In many M60-like domain-containing proteins have been detected CBM32, CBM5, CBM12, CBM52, BACON and PA14 domains. Most of 70% of M60-like domain-containing proteins were also predicted to possess a signal peptide (SP)

and one or more transmembrane domains (TMD), suggesting their extracellular or cell surface localization [41].

M60-like domain-containing proteins are shared by a large number of bacterial and eukaryotic microbes [PFAM database] [44] and are present in important mucosal microbes such as *T.vaginalis*, *Bacteroides thetaiotaomicro* , *Bacteroides fragilis, Bacteroides caccae, Bacillus anthracis, Clostridium perfringens, Vibrio cholera, Entamoeba histolytica, Cryptosporidium species,* suggesting that these proteins might play an important role in the biology of mucosal microbes as well as in host-microbial interactions (Figure I.3).

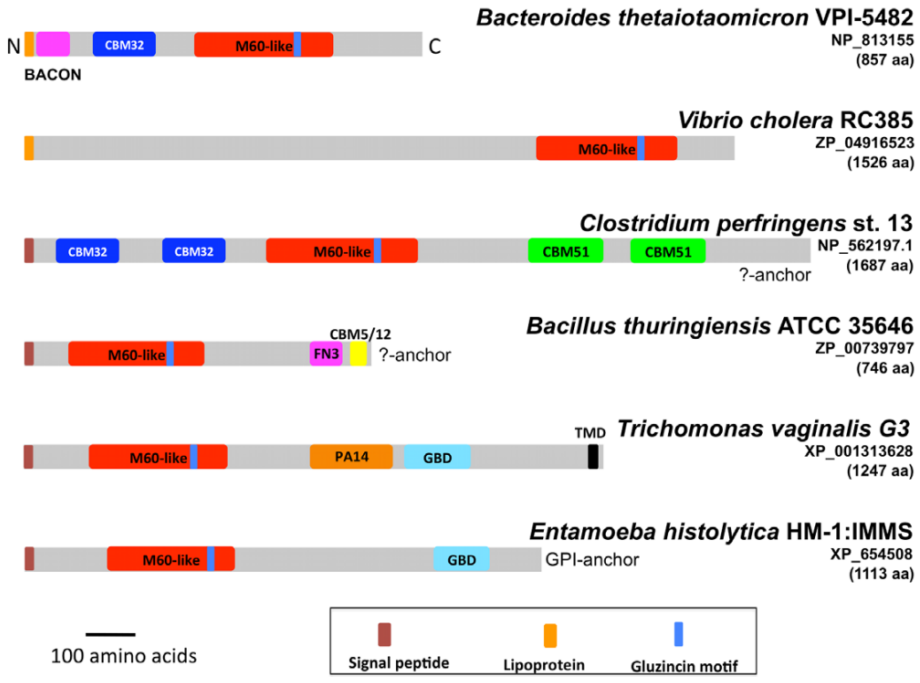


Figure I.3 - Structural organization of M60-like containing-proteins from different mucosal microbes.

The length of proteins and their structural features are shown for each pathogen, together with accession number. Species aligned to their N terminus. Several CBMs are associated with M60-like peptidase domains. CMB domains can be found at either the C-terminal or N-terminal side of the M670-like domain compared with the relative position of the protease domain, which is often conserved suggesting this configuration is functionally important. (Image form Nakjang et al, 2012).

I.5 Contact independent mechanisms

T.vaginalis pathogenic mechanisms involve also the contact-independent cytotoxicity. The pathogen is able to produce a wide range of hydrolase identified as cytoplasmic cysteine proteinase (20-100 kDa). These proteins are released by the parasite and have trypsin-like activity, functioning as cell-detaching factors (CDF) by degrading proteins of the ECM. CDF allow *T.vaginalis* to traverse the protective mucus barrier of host epithelium and aid in the release of host cells from tissue and mucosal desquamation [14, 45].
CDF levels have been shown to correlate with the severity of the clinical symptoms of vaginitis[2].
Another pathogenic mechanism used by *T.vaginalis* to damage target cell plasma membrane is the secretion of cytotoxic molecules such as perforin like activity, creating pores in erythrocyte membranes. *T.vaginalis* is also known to excrete different lytic factor having phospholipase A2 activities, to destroy nucleated cells and erythrocytes by degradation of phosphatidylcholine [14].
Recently, a new protein secreted by *T.vaginalis* was characterized. This protein is homolog of human macrophage migration inhibitory factor (MIF), a versatile proinflammatory cytokine involved in several processes, including immunity, cell proliferation and tumorigenesis. HuMIF is reported to be elevated in prostate cancer. As HuMIF, TvMIF inhibits macrophage migration, is proinflammatory and binds CD74 MIF receptor with high affinity. Moreover, it increases cellular proliferation and invasiveness of BPH-1 and PC3 *in vitro*. These data indicate that chronic *T. vaginalis* infections may result in TvMIF-driven inflammation and cell proliferation that contributes to the promotion and progression of prostate cancer [8].

I.6 *T.vaginalis* interacts with viruses and human-associated bacteria

The ability of *T.vaginalis* to interacts with viruses and human microbiota, including bacterial and fungal cells, constitutes a third pathogenic mechanism.
The parasite internalizes materials, e.g. lactoferrina and hemoglobin, through receptor-mediated endocytosis [12]. By endocytosis, *T.vaginalis* also interacts with

HIV, facilitating HIV entry and transmission in humans by damaging mucosal surface [9-11]. Recently it has been shown that the pathogen can also be infected by four dsRNA viruses (TVV) [46, 47].

T.vaginalis is capable to ingest different mammalian cells [28], including epithelial cells, immunocytes and spermatozoids, obtaining important sources of nutrient and contributes to defense from immune system. [12]

Moreover, the parasite phagocytes several types of bacteria colonizing the lower urogenital tract of human body, inducing an imbalance in the microbial community [48]. During bacterial vaginosis, for example, was observed a correlation between the presence of *T.vaginalis* and low abundance of protective lactobacilli and higher proportions of *Mycoplasma*, *Prevotella* and other bacteria typically involved in bacterial vaginosis [49]. Among several species of human macrobiota, *T.vaginalis* has shown a clinical association with two different species of Mycoplasma: *Mycoplasma hominis* [50], and *Candidatus Mycoplasma girerdii* [51] [52]. Mycoplasmas are the smallest self-replicating organisms lacking cell walls, have small genome and often dependent on their host. In the urogenital tract they are associated with bacterial vaginosis, pelvic inflammatory disease, preterm labor and preterm birth [52]. In spite of these associations with disease, *Mycoplasma hominis* and *Ureoplasma* are also commensal bacteria of the lower urogenital tract.

Acting as pathogen, *M.hominis* causes an infection linked with several pregnancy and postpartum complications like spontaneous abortion, endometritis and low birth weight [53-55], as for trichomoniasis.

Many pathogenicity mechanisms of *M.hominis* are not yet clear. The symbiosis between *T.vaginalis* and *M.hominis* is the only endosymbiotic relationship described so far involving two obligated human parasites that produce independent diseases in the same anatomical area. Previous studies have shown that Mycoplasma cells carried by *T.vaginalis* are able to infect human cells *in vitro*, suggesting that *T.vaginalis* could play a role of "Trojan horse" for the bacterium during infection [56]. However, the exact nature and fundamental aspects of this association still have to be cleared.

I.6 Object of this study

The overall aim of this study was to gain new insights into pathogenic mechanisms of *T.vaginalis*, focusing the attention on adhesion to host cells and interaction with human microbiota.

The specific objectives were as follows;

Characterize of putative M60-like domain-containing protease from *Trichomonas vaginalis* using biochemical approaches.

Analyse the functional aspects of CBMs present on putative M60-like domain protein of *T.vaginalis*.

Investigate the possible influence of *M.hominis* on pathobiology of *T.vaginalis*.

CHAPTER II

Characterisation of domains of putative *T.vaginalis* M60-LIKE protease

II.1 Introduction

Infections occur when the balance of host-pathogen interaction shifts to favour the pathogen. A multitude of virulence factors is used by pathogens to promote pathogenesis. Many pathogens facilitate their attachment to host tissue components by specific cell surface molecules, called adhesins. Other microbial pathogens secrete enzymes that digest host components to acquire nutrients and to inactivate host defence factors. Some pathogens are able to synthesize toxins that cause both the death of host cells and tissue damage. Several microorganisms express factors that inhibit specific host defence mechanisms or deregulate the host inflammatory response to their advantage. Moreover, many pathogens subvert host components to grow, survive and spread in the host environment. Among many components modulated by microbial pathogens, one common link is represented by heparan sulphate (HS) glycosaminoglycans (GAGs). GAGs are sugars attached to a protein backbone forming proteoglycans, a component of epithelial cell glycocalyx. Proteoglycans along with glycoproteins, glycolipids and transmembrane mucins, constitute glycocalyx of human cells [57]. All component of glycocalyx can act as receptor for microbial adhesion, while the entire structure represents an important second line of defence against invading pathogens, after mucosal surfaces [58].

II.2 Overview about HS-GAG

II.2.1 GAGs

GAGs are terminal carbohydrate structures in the extracellular matrix, comprised of repeating disaccharide units of hexosamine and uronic acid or galactose with various substitutions. GAGs are expressed widely in the human body and a number of bacteria, viral and parasitic pathogens exploit GAGs on key steps of pathogenesis, such as adhesion and invasion of host cells, cell-cell transmission and evasion of host defence mechanisms [59].

The list of GAGs includes heparan sulphate (HS)/heparin, chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), and hyaluronan (HA). Except for HA, all other polysaccharides are sulphated to various degrees and are covalently complexed to proteoglycans core protein (Figure II.1).

Among GAGs, HS can be considered a crucial common link that many pathogens exploited to infect human hosts and cause diseases, including those originating from STIs. Several studies have shown the multiple roles of HS in microbial invasion of human hosts [60]. A number of etiologic agents of STIs, such as HSV, HIV HPV and *Chlamydia trachomatis* express surface proteins that interact with HS to mediate their attachment to eukaryotic cells as a primary mechanism during mucosal infections [59, 60].

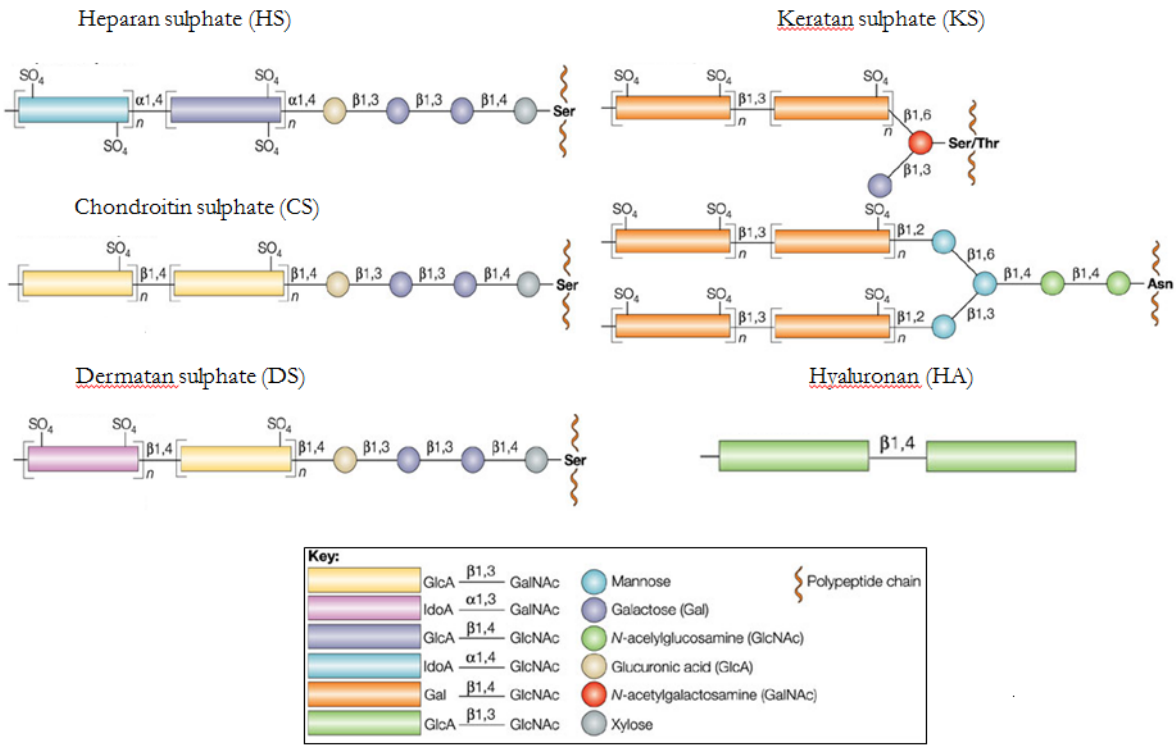


Figure II.1 - GAGs structure and synthesis.

Synthesis of HS, CS and DS start with addition of xylose to serin (Ser), proceeding by addition of two galactose residues and one glucuronic acid. In HS synthesis, there is an alternating addition of *N*-acetylglucosamine and glucuronic acid (GlcA), while for the assembly of CS and DS there is an alternating addition of *N*-acetylgalactosamine and GlcA. The polymerization of HS, CS and DS is completed by epimerization of GlcA to iduronic acid (IdoA) and sulphation (SO4) of different residues, resulting in a large micro-heterogeneity within the polymers. KS assembly is the result of alternating attachment of *N*-acetylglucosamine and galactose to O or N-glycans on the proteoglycan core and of sulphatation of several residues. HA is a large polymer of alternating *N*-acetylglucosamine and GlcA and is not attached to a core protein. (Image modified from Kleene R et al, 2004).

II.2.2 Heparin/heparan sulphate glycosaminoglycans

Heparin/heparan sulphate glycosaminoglycans, extracellular complex polysaccharides, are attached to a protein core or proteoglycan (HSPG) and are extruded by cells to the cell surface and into the extracellular space [61, 62]. Heparin (highly sulphated polysaccharide) and HS (the less sulphated polysaccharide), are negatively charged linear carbohydrate polymer composed of repeating uronic acid [d-glucuronic acid (GlcA) or l-iduronic acid (IdoA)] and 10-200 disaccharide units of D-glucosamine (GlcN) [61]. Variable patterns of substitution of the disaccharide units with N-sulphate, O-sulphate and N-acetyl groups give rise to a large number of complex sequences.

Heparin is commonly isolated from connective-tissue type mast cells and it is biosynthesized as heparin proteoglycan (Mr 750000–1000000). Multiple polysaccharide chains (Mr 60000–100000) are covalently attached to a unique proteoglycan called serglycin [61, 63]. Serglycin is a secretory vesicle proteoglycans implicated in inflammation, storing mast cell inflammatory mediators [63].

HS is also biosynthesized as a proteoglycan, but it has fewer and shorter polysaccharide chain than heparin proteoglycan [61]. HS proteoglycans are expressed and secreted by most mammalian cells, and are located on cell surfaces and in the extracellular matrix. HS is evolutionarily ancient and its composition has remained relatively constant from Hydra to humans [62]. Moreover, it is involved in important physiological functions, such as lipid metabolism, neurogenesis and cytokine/growth factor interaction [60].

One of the reasons that HS interacts with a diverse group of pathogens relates to the structural and functional diversity of HS originating from extensive modifications during its biosynthesis. The biosynthesis of HS occurs in the Golgi apparatus and is a sequential, multistep process (Figure II.2).

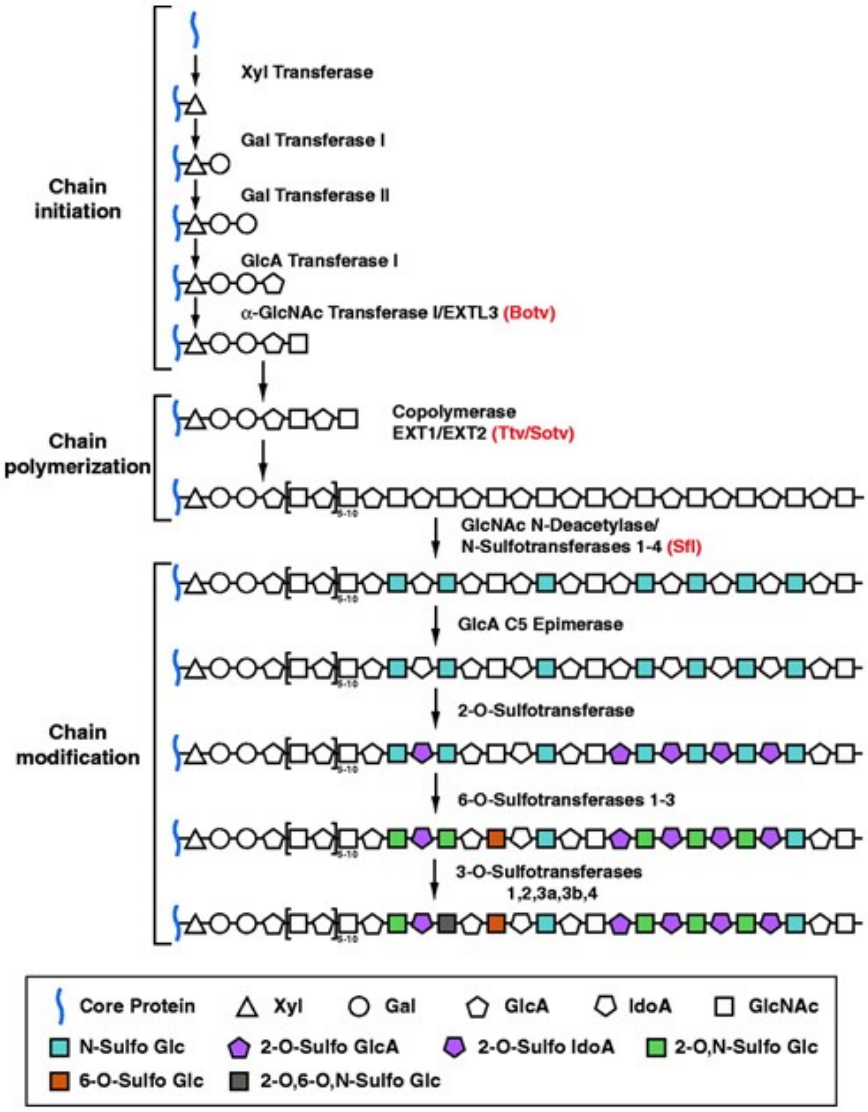


Figure II.2 - HS biosynthesis.

HS chains are synthesized on a core protein by the sequential action of individual glycosyltransferases and modification enzymes, in a three-step process involving chain initiation, polymerization and modification. Chain initiation: assembly of a linkage tetrasaccharide on serine residues in the core polypeptide. The tetrasaccharide is composed by GlcA-galactose (Gal)-Gal-Xylose (Xyl). This process is catalysed by four enzymes (Xyl transferase, Gal transferase I-II and GlcA transferase I), which add individual sugar residues sequentially to the non-reducing end of the growing chain. Chain polymerization: after the assembly of the linkage region, one or more α-GlcNAc transferases add a single α1,4-linked GlcNAc unit to the chain, which initiates the HS polymerization process. HS chain polymerization then takes place by the addition of alternating GlcA and GlcNAc residues, which is catalyzed by the EXT family proteins. Chain modifications: include GlcNAc N-

deacetylation and N-sulfation, C5 epimerization of GlcA to IdoA, and variable O-sulfation at C2 of IdoA and GlcA, at C6 of GlcNAc and GlcNS units, and, occasionally, at C3 of GlcN residues. The HS chains are ~100 or more sugar units long and have numerous structural heterogeneities Monosaccharaides are synthesized in the cytoplasm and transported into the Golgi, where they are used to synthesize a conserved tetrasaccharide, and then initiate and elongate heparan-sulphate-specific polysaccharide chains (Image from LinX, 2004).

II.2.3 HSPGs

HSPGs are glycoproteins, containing one or more covalently attached HS chains. There are two major subfamilies of cell surface HS proteoglycans (HSPGs), the syndecan (four members) and the glypicans (six members). The syndecan core proteins are transmembrane proteins and virtually all cell types express one or more syndecans [64]. The glypican core proteins are attached to cell membranes by a glycosylphosphatidylinositol (GPI) tail. Perlecan, agrin and collagen XVIII are HS proteoglycans but located in the extracellular matrix. These are also distinguished by their specific core proteins [65] (Figure II.3) (Table II.1 shows HSPGS classification depending on their localization).

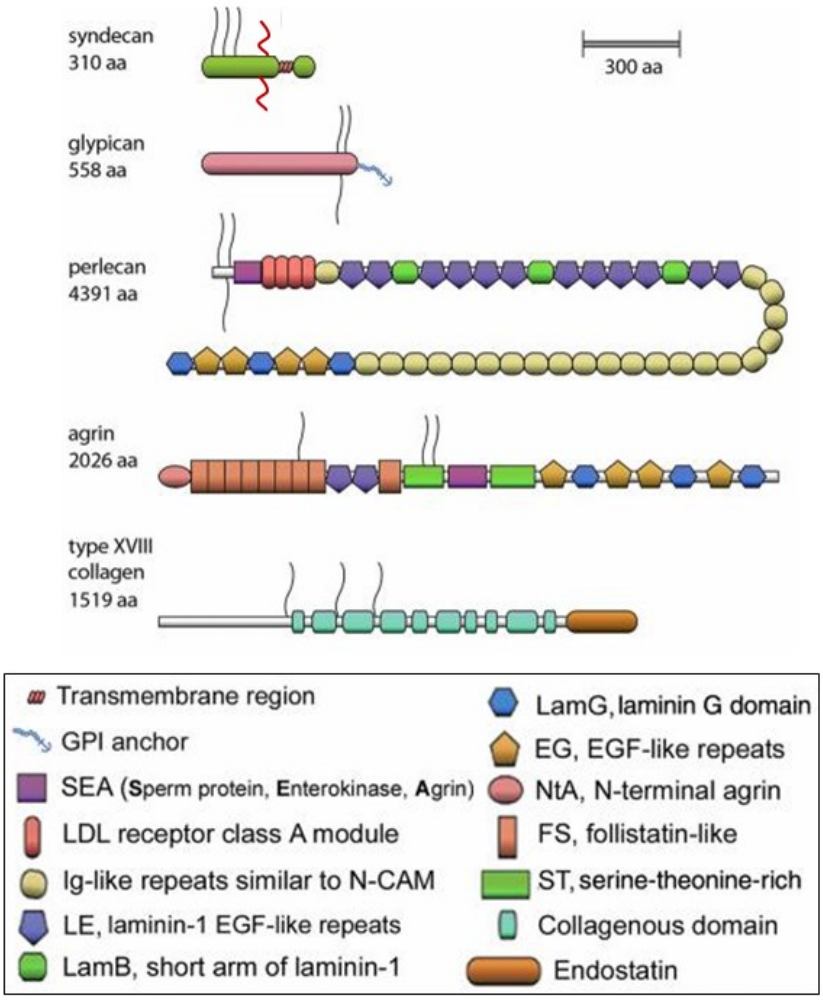


Figure II.3 - Schematic representation of major classes of HSPGs.

HSPGs include four syndecans, six glypicans, and one each of perlecan, agrin, and the hybrid HSPG/collagen type XVIII. The size is approximately proportional to the number of amino acid residues (aa). In the bottom panel there are various color-coded modules. HS chains are shown as black lines; CS chains are shown as red lines. (Image modified from Iozzo RV, 2001).

Table II.1 - Classification of HSPGs.

Class	*Proteoglycan*	*Core mass (kDa)*	*Chain type (number)*
Membrane-bound	Syndecan -1	31-45	3-4 HS
			1-2 CS
	Syndecan -2	31-45	2-3 HS
	Syndecan -3	31-45	3-4 HS
			1-2 CS
	Syndecan -4	31-45	2-3 HS
	Glypican-1,	57-69	1-3 HS
	Glypican -6		
	Betaglycan	110	1-2 HS
			1-2 CS
	Neuropilin-1	130	1 HS or CS
	CD44v3	37	1 HS
Secretory vesicles	Serglycin	10-19	10-15 Heparin
			10-15 CS
Extracellular matrix	Perlecan	400	1-4 HS
	Agrin	212	2-3 HS
	Collagen XVIII	150	1-3 HS

HSPGs have multiple activities in cell and tissue, such as to collaborate with other matrix component to define basement membrane structure and to provide matrix for cell migration (perlecan, agrin and collagen XVIII); to maintain proteases in an activate state; and to regulate various biological activities after secretion (serglycin). HPGSs protect cytokines, chemokines, growth factors, and morphogens by binding and act as receptors for proteases and proteases inhibitors, regulating their spatial distribution and activity. Moreover, syndecans and glypicans can cooperate with integrins and other cell adhesion receptors, allowing cell-ECM attachment, cell-cell interactions, and cell motility. They can also act both as endocytic receptors for clearance of bound ligands and as coreceptor [64]. The coreceptor function of HSPGs aids the formation of ligand-receptor complexes either through conformational change of ligand and/or receptor or using themselves as a template to approximate ligand and

receptor [64]. The binding takes place both through core protein interaction and through GAG chains modified. One feature shared by many coreceptors is modification of GAG chains, which contribute into interaction between proteoglycans and ligands, extracellular matrix proteins or other cell surface receptors, regulating cell adhesion, migration and invasion also in the human diseases. The most common modifications occur on one or more site of HS and CS chains.

HSPGs signaling coreceptors include CD44, glypicans (Glypicans-1, -6), neurophilins, syndecans (Syndecan -1, -4) and betaglycan.

All transmembrane proteoglycans signalling co-receptor, such as transmembrane syndecans, undergo ectodomain shedding resulting in proteolytic cleavage of core protein near the transmembrane domain that allows releasing of the extracellular domain from the cell surface. The soluble extracellular domains of HSPGs continue to carry their glycosaminoglycan modifications [66]. Syndecan shedding is mediated by matrix metalloproteinases (MMP1, MMP7, MMP9, ADAM17) [67, 68] but even some microorganisms can enhance host cell proteolytic shedding of syndecan-1 (Syd-1), with an increase of bacterial colonization [69, 70]. Moreover, it has been shown that the amount of HS presents on syndecan core proteins regulates both the rate of syndecan shedding and core proteins synthesis [71].

II.3 Syndecan families

Syndecans family is a group of transmembrane HSPGs with a long evolutionary story. The four mammalian members, syndecan-1 to syndecan-4, are composed by core protein with covalently attached GAG chains. The syndecans contain an N-terminal extracellular domain or ectodomain, a hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain. The ectodomain contains three consecutive consensus Ser-Gly sequences for HS chain attachment close to N terminus, and Ser-Gly sequences for CS chains at site near the plasma membrane [72]. The length of transmembrane and cytoplasmic domain is highly conserved among member of syndecans family, whereas the length of the ectodomains varies considerably. Many syndecan roles are attributed to their HS

chains, which interact with a wide range of ECM and adhesion molecules, chemokines and cytokines [72]. The functions of syndecans include anchorage of cells to ECM with associated HS binding domains; maintenance of epithelial and endothelial morphology; binding to and modulation of activity of HS binding growth factors; modulation of activity of several proteases and their inhibitors; and signaling molecules [69, 72]. Moreover, syndecan family has been proposed to act as adhesion and internalization receptors for pathogenic microorganisms.

By shedding, the syndecan ectodomains complete with their GAG chains, are released from the cell surface forming soluble ectodomains that may function either as paracrine or autocrine effectors, or competitive inhibitors of intact proteoglycans [73, 74]. These ectodomains are in fluids accumulating, following injury and inflammation. Matrix metalloproteinases (MMPs) and membrane type MMPs (MT1-MMPs) are involved in the shedding and catabolic process of syndecans, whereas heparanase and endosulfatases modify HS chains within extracellular environment, including the ability of HS chains to bind and sequester growth factors [73] [75]. Moreover, a recent study has shown that the loss of HS chains enhances both the susceptibility of core protein of Syd-1 to proteolitic cleavage by matrix metalloproteinases and a dramatic increase in core protein synthesis [71].

In the table II.2 have listed the MMPs involved in syndecan cleavage both *in vitro* and *in vivo* [68, 73]. Moreover, MMP2, MMP7 and MMP9 bind HS chains of syndecan.

Table II.2 - List of MMPs involved in syndecan cleavage *in vitro* and *in vivo*.

	Syndecan-1	Syndecan-2	Syndecan-3	Syndecan-4
MT1-MMP	✓			✓
MMP2 (gelatinase)	✓	✓		✓
MMP3	✓			
MMP7 (metrilysin)	✓			
MMP9 (gelatinase)	✓	✓		✓

II.3.1 Syndecan-1 shedding is implicated in microbial pathogenesis.

Syd-1 shedding is one of the general host responses to tissue injury and inflammation [69]. Moreover, the activation of Syd-1 shedding is used by some pathogens as an important virulence mechanism [69, 70, 76]. For example, *P. aeuroginosa* actives Syd-1 shedding through LasA, a virulence factor for its lung infection and *S.aureus* sheds syndecan-1 through α- and β–toxins, implicated in sthaphylococcal infections. Both of these microorganisms activate a protein-tyrosine kinase (PTK)2 –dependent intracellular signalling mechanism to stimulate the ectodomains cleavage [69, 76]. *Streptococcus pneumonia*, unlike *P. aeuroginosa* and *S.aureus*, directly sheds Syd-1 ectodomain through zinc metalloproteinase (ZmpC) [70]. So far, it is not completely clear how Syd-1 shedding promotes microbial pathogenesis.

Moreover, Syd-1 has been identified as a substrate of MT1-MMP that cleaves the Gly82-Leu83 and Gly245-Leu246 bonds of a recombinant syndecan-1 fusion protein [68]. Increased levels of shed Syd-1 are present in sera of patients with some cancer types [71]. Moreover, shed Syd-1 plays an active role in driving tumour progression, stimulating signalling and proliferation in tumour cells, and

enhancing angiogenesis, osteolysis, growth, and spontaneous metastasis of tumour cells [73].

II.4 Overview on matrix metalloproteinases

Matrix metalloproteinases, also called matrixins, are the major enzymes implicated in cleavage of several ECM constituents, as well as non-matrix proteins, regulating cell-matrix composition [77]. In the MEROPS database, MMPs are classified as the metzincin subfamily of zinc metalloproteases family M10. This family is distinguished by a highly conserved motif, HEXXHXXGXXH, where histidines (H), glutamic acid (E) and glycine (G) residues are invariant. The histidines bind zinc at the catalytic site. A conserved methionine also presents in the catalytic domain forms a "Met-turn", eight residues after the zinc binding motif, forming a base to support the structure around the catalytic zinc. A third zinc binding ligand differentiates the zincin clan further into subclans e.g gluczincins (E), aspzincins (D) or metzincins (H/D) [77].

Metzincin subfamily is further subdivided into four families: serralysins, astacins, ADAMs/adamalysins and MMPs.

The 23 human MMPs typically containing a signal peptide (SP), a propeptide of ~80 amino acid, a catalytic domain of ~170 amino acid, a linker peptide of variable lengths, and a hemopexin (Hpx) domain of ~200 amino acids, with exceptions of MMP-7 (matrilysin 1), MMP-26 (matrilysin 2) and MMP-23, which lack or present changes of domains in the structure [78].

With exception of six membrane -anchored MMPs, the remaining 17 MMPs are destined for secretion into the extracellular milieu [79].

MMP14, a membrane-type MMP (MT1-MMP), exists in a membrane-bound rather than a secreted form [80]. It is expressed on endothelial cells, on fibroblasts, on osteoclasts, and on monocytes. Moreover, MMP14 has been also found to be produced on cancer cell membranes. [81]. The Hpx domain is the region responsible for binding to and shedding of CD44 that leads to rearrangement into intracellular cytoskeleton and inducing the cell migration and invasion processes [82].

MT1-MMP has a wide substrate specificity, such as aggrecan, elastin, perlecan, and fibronectin; and cleaves and promotes shedding of Syd-1 [68] , of betaglycan [83] and of MUC1, a transmembrane mucin with a critical role in embryo implantation, protection of mucosal epithelial cells from microbial and enzyme attack, and in several aspects of tumour progression [84].

II.4.1 M60-like domain containing proteins of *T.vaginalis*

Recent study has shown a novel protein domain termed 'M60-like (PF13402) domains', shared by several bacterial and eukaryotic mucosal microbes [41].
The presence of the extended consensus HExxHxE in this domain suggested that the M60-like domain containing proteins could be considered as gluzincin metallopeptidases processing extracellular glycoprotein targets. This hypothesis is further supported by evidence that M60-like domain containing proteins represent distant relatives of viral-enhancin proteases known to degrade insect mucins. Moreover, Dr.Didier Ndhe in his PhD thesis has shown that three M60-like domains proteins of *Bacteroides thetaiotaomicron*, a bacterial gut mutualist of human body, exhibited mucin protease activity. This proteolytic activity was shown to be inhibited in a mutant version of the protein as well as in the presence of Ethylenediaminetetraacetic acid (EDTA), implying BT4244 and its relatives are metal dependent proteases. The CBMs contained in *B. thetaiotaomicron* are from family 32 and are capable to bind galacto-configured sugars that are common to mucin glycans.
A total of 25 TvM60-Like containing proteins were also identified in *T.vaginalis* and 11 of these possess XExxHxE motif. Among them, six have one TMD with only three of these entries possessing a complete M60-Like domain. A sequence alignment suggested that most entries are likely represented gene fragments or truncated version of longer proteins [41]. Among three complete TvM60-like containing proteins, i.e. TVAG339720, TVAG189150 and TVAG199300, TVAG339720, TVAG189150 were detected on the cell surface by proteomic analysis, and TVAG339720 is the only one identified in all six tested isolates [38] (figure II.4). Moreover, native TVAG339720 and TVAG189150 proteins had been detected in *T. vaginalis* cell membrane extracts and the extracellular

localisation of the TVAG339720 protein has indeed been confirmed through immunofluorescence assays by our collaborators in the U.S.A (personal communication, Prof. Robert Hirt).

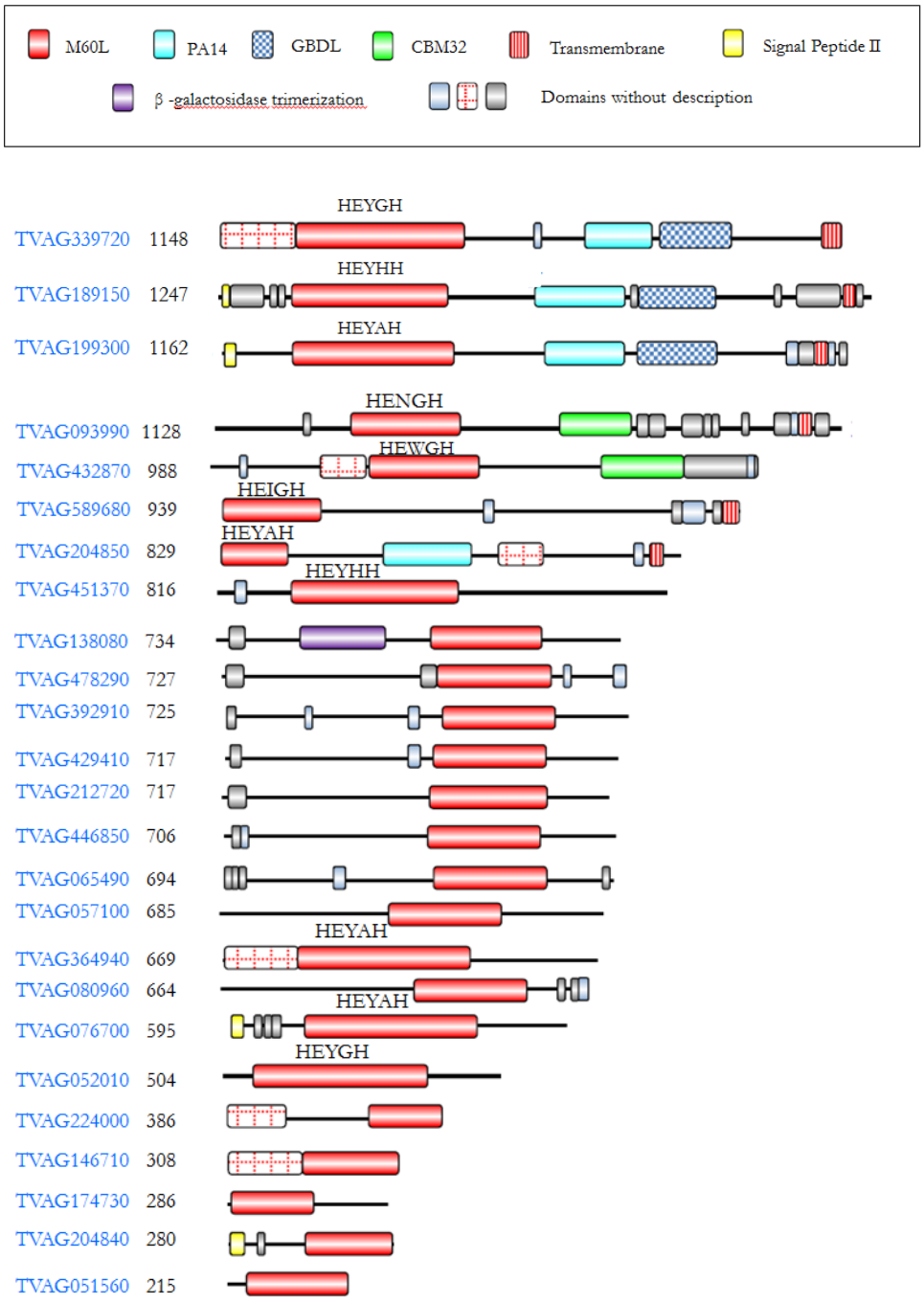


Figure II.4 - Domain architecture of *T.vaginalis* M60L entries from PFAM database.

The first putative protein (TVAG339720) is that under biochemical characterisation in the current study. (Image modified from Dr.Ndhe PhD thesis).

Using HMM profile-profile searches, PA14-like and CBM32 or galactose-binding domain (GBD) like sequences were identified in M60-like proteins from *T.vaginalis*. These domains were also detected in several M60-like domain-containing proteins of other mucosal microbes, such as *C.perfringens and C.albicans,* to target galacto-configured sugars [85] [86]. In Dr. Ndeh thesis is shown that TVAG199300-M60L failed to degrade any of the mucin substrates tested against the protein and the PA14 domain of TVAG339720 binds heparin instead of mucin glycans.

The preference for highly sulphated heparin was an indication that sulphate groups may play a role in heparin recognition by the PA14 domain.

Heparin is a GAG, structurally similar to heparan sulphate. These GAGs form part of well-known epithelial cell surface proteoglycans, such as syndecans and glypicans [64]. These proteoglycans along with mucins, glycoprotein and glycolipids constitute epithelial cell glycocalyx at mucosal surfaces [57].

An important hypothesis is that the actual targets for the TvM60 proteins containing PA14 domains of *T.vaginalis* are heparan sulphate glycosaminoglycans. Data accumulated so far about M60-like domain-containing proteins of *T. vaginalis* suggest that TVAG339720 and its close relatives may represent an important virulence factors for the organism.

II.5 Objectives

In this chapter the aim of work was tested the hypothesis that TVAG339720, a M60-like domain-containing proteins of *T. vaginalis,* is a glycoprotein targeted extracellular zinc-metalloproteases.

Experiments were performed to study proteolytic and carbohydrate binding activity of TvM60L, PA14-GBDL and GBDL domains of *T. vaginalis* M60-like domain-containing protein.

II.4 Materials and methods

II.4.1 Materials

Heparan sulphate, 10 mg, was obtained from Celsius Laboratories. Plasmin ≥2.0 units/mg protein (human plasma fibrinolysin, EC 3.2.21.7) was obtained from Sigma-Aldrich. Recombinant human Syndecan -1/CD138 was from R&D.

II. 4.2 Bacterial strains

Two different *Escherichia coli* (*E.coli*) strains were used to investigate domains from TVAG_339720: DH5α and Tuner strain. The futures are listed in the table II.3 below.

Table II.3 - Bacterial strain used in this study

Strain	***Genotype***	***Use***	***Reference***
DH5α	F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+) phoAsupE44 -thi-1 gyrA96 relA1	DNA cloning	Woodcock, D.M et al, 1989
Tuner (DE3)	F – ompT hsdSB(rB– mB–) gal dcm lacY1 (DE3)	Protein expression	Novagen

II.4.3 Plasmids

The plasmid pET28a (Novagen) was used for expression of different domains from M60-Like zinc-metallopeptidase of *T.vaginalis*.
This bacterial expression vector of ~5.4Kb contains a T7lac promoter and a natural promoter and coding sequence for the lac repressor (lacI). Moreover, it carries an N-terminal His tag, thrombin cleavage site, internal T7 epitope tag, C-terminal His tag; kanamycin resistance and restriction enzyme cloning.
The map of pET28a is in appendix C.

II.4.3 Growth and selective media

During this study were used two different growth media: Lurie-Bertani (LB) medium and LB-agar. LB medium is composed by Bacto®tryptone (10g/L), Bacto®yeast extract (5g/L) and NaCl (10g/L). The components were dissolving in 1L of water and the pH of solution was adjusted to 7.4 with NaOH.
LB-agar was prepared added 2g of agar (add information about it) to 100ml of LB medium (2%). All media were sterilized by autoclaving at 121°C, 32 lb / inch -2 for 20 min. After autoclaving, LB medium and LB-agar were left to cool to between 45°and 50°C and then a specific antibiotic was added. For our study we used kanamycin antibiotic in a final concentration of 20μg/ml.
LB-agar mixed with antibiotic was poured into 90mm sterile petri dishes and storage at 4°C after solidification.

II.4.4 Sterilization

Different sterilization methods were adopted, depending on materials.
Media, laboratory glassware and some solutions were sterilized by autoclave (Prestige Medical), using steam under high pressure (32 lb / inch -2) at 121°C for 20 min.
Sterile syringe (Plastipak®,Becton Dickinson) and appropriate pore-sized (0.22-1μm) Millipore filter discs (Supor® Acrodisc®) were adopted to sterilize solutions impossible to autoclave.

II.4.5 Plating bacteria

A single inoculum of bacteria suspension (150ul) was spread over the surface of LB-agar plate.
A glass spreader, earlier flame sterilized by dipping in alcohol (100% EtOh) and cooled for about a minute, was placed in contact with the inoculum and was used for distribution and absorption of bacteria suspension into the agar. Plates

were allowed to dry at room temperature and later incubated in an inverted position at 37°C for overnight growth in an incubator. (Name of incubator)

II.4.5 Storage of bacteria and DNA samples

Bacteria colonies growth on LB-agar plates was stored in a fridge at 4°C for a maximum of one week. Plasmids were stored at -20°C in water.

II.4.6 Determination of DNA and protein concentration

The concentration of DNA and proteins was quantitated by measuring the absorption of ultraviolet light at 260nm (for nucleic acid) and 280nm (for protein) using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc, USA).
The Beer-Lambert law:

$$A=\varepsilon CL$$

where A is the absorbance at a given wavelength of light (nm), ε is the molar extinction coefficient, C is the molar concentration of sample, and L is the length of the light path (cm), was used to calculate the specific concentration of DNA and protein starting from absorption value at 260nm (A260) or at 280nm (A280).

II.4.7 Primers

Different primers were designed for cloning and sequencing experiments during this study. The lengths of oligonucleotides were between 20 to 40bp and the melting temperatures (Tm) were greater to 50°C, to have the best chance for maintenance of specificity and efficiency during annealing step.
Primer parameters were evaluated using the online Oligonucleotide Properties Calculator tool at http://www.basic.northwestern.edu/biotools/OligoCalc.html and Tm of different sets of primers were calculated by

$$Tm = 64.9°C + 41°C \times (\text{number of G's and C's in the primer} - 16.4)/N$$

where N is the length of the primer.

Primers developed for cloning experiments were designed adding CCGCG, CCGG or CGCG spacer plus restriction sites to the 5'-ends, to allow the cleavage by restriction enzymes and then the ligation in vectors cut in the same sites.

Primers for cloning were synthesized dry by Sigma (Sigma Aldrich, UK) and resuspended in distilled water to the desired concentration.

II.4.8 Polymerase chain reaction (PCR)

Amplification reactions of the DNA under investigation were performed by PCR technique (Mullis & Faloona, 1987), using the Novagen Hot start PCR kit (Novagen) and PHC-3 thermocylcer (Biorad). A typical PCR reaction set up and the standard program used are schematised as shown in the tables II.4 and II.5 below.

Table II.4 - Typical PCR reaction set-up.

Components and concentrations	***Volume***
Autoclaved distilled water	19µl
10 x KOD buffer minus Mg^{2+} (10 x)	5µl
dNTP's (2 mM)	5µl
Q-solution (DMSO)	5µl
$MgSO_4$ (25 mM)	4µl
Template DNA (~70 ng/µl)	1µl
Novagen ® KOD DNA Polymerase (2.5 U/µl)	1µl
Forward oligonucleotide (5 µM)	5µl
Reverse oligonucleotide primer (5 µM)	5µl
Total volume	**50µl**

Table II.5 - Typical PCR reaction program.

Program name	*Event*	*Temperature*	*Duration*	*Number of Cycles*
Program 1	Denaturation	95 °C	1 min	1
Program 2	Denaturation	95 °C	1 min	30
	Annealing	50 °C	1 min	
	Extension	68 °C	1min/1kbp fragment size	
Program 3	Polishing	68 °C	10 min	1
Program 4	Storage	10°C	≤ 24hr	1

II.4.9 Agarose gel electrophoresis for analysis or PCR products

Agarose gel electrophoresis (AGE) (Meyers et al, 1976) is a technique used for detection and separation of DNA molecules. In this study, AGE was used to analyse the presence or absence of PCR products, of plasmid DNA purified from bacteria, and to check plasmids digested by restriction enzyme (Figure II.5).

The gel was prepared by dissolving of 0.5g of low grade (MELFORD Ltd) or SeaKem® Gold Agarose (Lonza) in 50 ml of TAE (Amersham) in a 200 ml conical flask to obtain a 1 % agarose solution. The solution was mixed, heated in in a microwave oven at 450 watts for about 5min and allowed to cool to about 50°C. After cooling, 5µl (1mg/ml) of an ethidium bromide solution was added to the solution, which was stirred again and poured into a gel casting mould.

After solidification, the gel was put in electrophoresis equipment [HU10 Mini Plus Horizontal Gel Unit (SCIE-PLAS Ltd) and BDH horizontal gel mould connected to a Bio-rad Mini-PROTEAN® Tetra Cell power supply (Bio-rad)] and submerged in 50 ml of TBE buffer.

PCR samples (5µl) were mixed with 2µl of DNA loading buffer [0.25% (w/v) bromophenol blue, 50% (v/v) Glycerol, 10 x TBE buffer (8.9 mM Tris base, 8.9

mM Boric acid, 2 mM EDTA pH 8.0)] and were applied into the gel, alongside a standard (3µl of Bioline HyperLadder™ I markers).
To run an AGE experiment, (70V Biorad machine) Amersham machine was used to constant voltage (100V) for about 1h and the results were visualized in the UV range by Bio-Rad Gel Doc 1000 system (Bio-Rad).

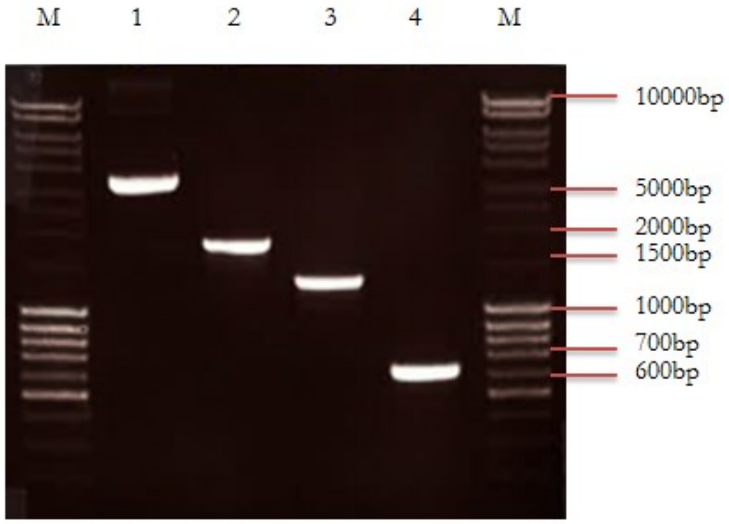


Figure II.5 - PCR products analysed by AGE.
Vector pET28.a (lane 1) and PCR products of TvM60L (lane 2), TvPA14-GBDL (lane 3) and TvGBDL (lane4) were analysed on a 1% of agarose in TAE buffer. Lanes M: Bioline HyperLadder™ I standards (3µl). Lanes 1-a: AGE results from 5 µl of different PCR reactions.

II.4.10 PCR products purification

The kit QIAquick PCR Purification Kit (Qiagen) was used to purify PCR products as described in manufacturer's instructions.

II.4.11 Digestion with restriction enzymes

Digestion reactions to PCR products and plasmids containing cleavage sites for restriction enzymes were made in four steps:

1. Digestion with first restriction enzyme
2. Sample purified by QIAquick PCR Purification Kit (Qiagen) and eluted with 30µl of distillate water
3. Digestion with second restriction enzyme
4. Sample purified by QIAquick PCR Purification Kit (Qiagen) and eluted with 10µl of distillate water

Digestion reactions were incubated in a water bath at 37°C for1.5h. The amount of restriction enzymes was of 15unit (U) and one unit of enzyme is defined as the amount of enzyme required to cleave 1µg of DNA in 1 h at 37 °C. Buffers and restriction enzymes used in this work were ordered from Fermentas (MBI Fermentas, UK).

In the table II.6 is shown a typical set-up of a digestion reaction.

Table II.6 - Set-up of a typical restriction enzyme digestion reaction.

Components	***Volume***
Distilled water	1-2µl
DNA fragment / plasmid(~0.1 – 0.5µg)	50µl
Restriction enzyme buffer (10 x)	6µl
Restriction enzyme (10U)	2-3µl
Total volume	**~60ul**

II.4.12 Ligation reactions

The ligation reactions involve plasmids and PCR products (insert) after digestion with restriction enzyme. Most of these enzymes are able to digest DNA asymmetrically across their recognition sequence, in order to obtain overhangs called "sticky ends". These sticky ends allow the vector and the insert to bind to each other. Before ligation, the concentration of cut inserts and vectors was determined and data were used to set-up the reactions. The molar ratio of insert

to vector is usually used at around 3:1.The ligation reactions were incubated for at least 1h, at 37°C.
In this study, Rapid DNA ligation KIT (Fermentas Life Science, UK) was used to perform ligation reactions. In the table below (table II.7) is schematized an example of ligation reaction set-up.

Table II.7 - Set-up of a ligation reaction.

Components	***Volume***
Vector (10 ng/µl)	2µl
Insert DNA(10 ng/µl)	6µl
5x Ligase buffer	4µl
T4 DNA Ligase(4 U/µl)	1µl
H2O (Nuclease free water)	7µl
Total volume	**20µl**

II.4.13 Transformation of competent *E.coli* cells

Chemically competent cells stored in -80°C, were taken from freezer and allowed to thaw on ice for about 5 min. After defrosting, 5µl of plasmid or ligation were mixed with bacteria and allowed on ice for further 1h. Soon after, bacteria were heat-shocked by incubation in a Techne Dri-Block™ DB-2A at 42 °C for 2min and were immediately replaced on ice for ~ 3 min.
Transformed *E.coli* cells were plated on LB-agar added with appropriate antibiotic (Section I.3 and I.5) and plated cells were growth overnight at 37°C.

II.4.14 Plasmid DNA purification by mini prep

Plasmid purification includes three steps: growth of bacterial culture, harvesting and lysis of the bacteria, and purification of the plasmid DNA.
Single colonies of transformed *E.coli* cells grown overnight on selective LB-agar, were subcultured overnight, into 5ml or 10ml of LB with appropriate antibiotic at 37°C with shaking at 180 rpm.
Following overnight growth, bacteria were used for plasmid extraction by Qiagen QIAprep ® Spin Miniprep Kit (Qiagen), in accordance with manufacturers' instruction.

II.4.15 DNA sequencing

Plasmids DNA were sequenced using Sanger sequencing services (GATC Biotech AG, European Custom Sequencing Centre, Cologne, Germany) to check cloned DNA sequences. The samples, with concentration between 30ng/μl and 100ng/μl, were put in a 1.5ml Eppendorf tube and labelled with pre-ordered sequencing labels before posting to GATC Biotech.
The primers used for sequencing were either custom-designed or standard sequencing primers available from GATC Biotech website. In this study, standard primers were T7- (TAATACGACTCACTATAGGG) and reverse primers (CTAGTTATTGCTCAGCGGT) complementary to regions within plasmids. DNA and primer custom- designed were sent in labelled separate tubes with total volume of 20ul each, sufficient for up to 8 reactions. Multiple sequence alignment tools such as Multalign (http://multalin.toulouse.inra.fr/multalin/) were used to analyse sequencing data, by alignment with the original DNA sequence.

II.4.16 Induction of recombinant proteins expression in *E.coli* and cell lysis

For recombinant protein expression, *E.coli* Tuner (DE3) strains were transformed with 1µl of sequenced recombinant plasmids and afterwards plated on appropriate selective media for overnight growth at 37°C. The next day a single colonies harbouring plasmids were picked, inoculated into 10ml of LB containing antibiotic and was grown overnight at 37°C with shacking (180rpm).
Thereafter, bacteria grown during the night were inoculated in 1L or 2L of LB plus antibiotic in 1L flasks and were grown at 37°C with aeration (180rpm) until an OD600nm between 0.6 and 1. Before induction with 0.1mM IPTG, cells were cooled under running tap water to about 16°C and were grown overnight at the same temperature before protein purification.
For protein purification, cells were harvested in 500 ml centrifuge pots (Nalgene) by centrifugation at 5000 × g for 10 min at 4°C using a JA-10 rotor of a Beckman J2-21 centrifuge (Beckman Coulter, Inc.). After discharged of supernatants, pellet fractions were resuspended in 7ml of Talon buffer (20 mM Tris/HCl pH 8.0 plus 100 mM NaCl) per 400ml of original culture volume.
Cells were then lysed by sonication for 45sec on ice for twice; using a B. Braun Labsonic U sonicator (B. Braun, Melsungen, Germany) set at low intensity (~45 watts) and soon after transferred into 50ml centrifuge tubes (Nalgene) for centrifugation. JA25.5 rotor in a Beckman J2-21 centrifuge (Beckman Coulter, Inc.) was used to centrifuge lysed cells at 19000rpm for 10-20min at 4°C. The supernatant or cell free extract (CFE or soluble fraction) was collected for protein purification.
After centrifugation steps, a small amount of pellet fractions were collected, resuspended in 50µl of Laemmli sample buffer 1x, boiled at 100°C in a boiling water bath for 5 min and stored at room temperature, for later analyses.

II.4.17 Purification recombinant N-terminal 6x His-tagged proteins

Recombinant N-terminal 6xHis-tagged proteins from CFE were achieved by immobilised metal affinity chromatography (IMAC), using NiNTA Agarose resin (Quigen), containing Nickel Nitrilo-triacetic Acid. Histidine residues in the His-tag bind to the vacant positions in the coordination sphere of the immobilized nickel ions with high specificity and affinity. Briefly, columns were filled with a 2.5 ml bed volume of NiNTA resin and equilibrated with at least 7 ml of Talon buffer (20 mM Tris/HCl pH 8.0 plus 100 mM NaCl).
The CFE solution was then applied onto the resin bed in the column and allowed to drain by gravity. The flow through (FT) was collected and saved for later analyses. The resin was washed with 5 ml of Talon containing 25mM of imidazole. Elution of the bound protein from the resin was achieved by sequential application of 5 ml volumes of Talon buffer containing 50mM, 100mM, 150mM and 250mM of imidazole. All eluted fractions were collected and saved for subsequent analyses by SDS PAGE or for further purification.

II.4.18 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE, technique described by Laemmli in 1970, was used to analyse protein expression. In the table II.8 are described solutions and buffers prepared for SDS-PAGE experiments.
3µl of pellet fractions, 10µl of CFE, FT, 25mM, 50mM, 100mM and 250mM fractions and a protein standards were applied on 10% of polyacrylamide gels (Acrylogel 3; BDH Electran®) using Bio-rad Mini-PROTEAN® Tetra Cell system (Bio-rad) according the manufacturer's instructions. The use of protein standards (PageRuler Prestained Protein Ladders, Thermo Scientific) was necessary to estimate the protein molecular weight after staining.
SDS-PAGE was run at 150 volts and InstantBlueTM stain (Expedeon) was used to stain gels after electrophoresis for at least 10min. Following, gels were washed and left overnight in distilled water, to allow the image acquisition using a Canon PowerShoot A75 camera (Canon).

Table II.8 - Solutions and buffer used for preparation of SDS-PAGE.

Component	***Volume/Amount*** ~For 4 gels
Resolving gel (10%)	
0.75 M Tris/HCl buffer, pH 8.8 with 0.2 % SDS	7.5ml
40 % Acrylamide (BDH Electran acrylamide, 3 % (w/v) bisacrylamide)	4.6ml
d.d. H2O	2.8ml
10 % (w/v) Ammonium persulphate	72µl
TEMED	24 µl
Stacking gel	
0.25 M Tris/HCl buffer, pH 8.8 with 0.2 % SDS	3.75ml
40 % Acrylamide (BDH Electran acrylamide, 3 % (w/v) bisacrylamide)	0.75ml
d.d. H2O	3.0ml
10 % (w/v) Ammonium persulphate	60µl
TEMED	20µl
Sample/Loading buffer	
SDS	10% (w/v)
0.25 M Tris/HCl buffer, pH 8.8 with 0.2 % SDS	5ml
Glycerol	25% (w/v)
β-mercaptoethanol	2.5ml
Bromophenol blue dye	0.1%
Running buffer	
32 mM Tris/190 mM glycine, pH 8.3	350ml
SDS	0.1%

II.4.19 Western blotting

Western blotting was carried out using the Biorad Trans-Blot Turbo Transfer System (Bio-rad). Nitrocellulose (NC) membranes, used for the transfer electrophoresed proteins, and filter papers were soaked in transfer buffer for 3min and put together to obtain a transfer sandwich. In accordance with manufacturers' instruction of blotting equipment, the sandwich was achieved putting two filter papers at the bottom, followed by NC membrane, the gel and other two filter papers. Blotting was performed at 25 V for 30 min.

II.4.20 Ion-exchange and concentrating proteins

Protein samples were dialysed overnight at 4°C, into 50mM of NaH_2PO_4, pH7.
For the protein concentration, Vivaspin™ centrifugal filter concentrators (VivaScience) were routinely used. Briefly, protein sample were transferred into concentrators with the appropriate molecular weight (5, 10 or 30 kDa) cut-off filter, followed by centrifugation at 3000 × g, at 4°C, for about an hour using a swing bucket type - MSE Mistral 3000i bench centrifuge (MSE, UK). This step could be repeated several times to obtain higher protein concentrations.

II.4.21 Isothermal titration calorimetry (ITC)

The binding of recombinantly expressed putative carbohydrate binding modules to sugars was assessed by Isothermal Titration Calorimetry (ITC), using a MicroCal™ VP-Isothermal Titration Calorimeter (Microcal, USA).
After dialysis overnight against a buffer of choice, recombinant proteins were filtered alongside the dialyses buffer using a sterile 1.2 μm filter (acrodisc). The filtereds dialysis buffer was used to dissolve sugars to be tested. Dissolving in the same buffer helps to minimize heats of dilution during titration into the recombinant protein. After degassing, filtered proteins and sugars were applied to the micro-calorimeter according to the manufacturers' instructions.

Typically 27 injections (10µl per injection) of the degassed ligand into the protein solution in the reaction cell were made with rapid stirring (307 rpm), at 300 s intervals. Following each injection, the heat generated (in exothermic reactions) or absorbed (as in endothermic reactions) due to the interaction of the protein with the ligand is calculated from the electrical power required to maintain the temperature of the reaction cell against that of the reference cell.
MicroCal Origin software (version 7.0) is used to fit data, by non-linear regression and applying a simple one-site binding model yielding the association constant (Ka), stoichiometry of binding (n), the enthalpy of binding (ΔH) and the entropy of binding (ΔS).
Ka, n, ΔH and ΔS were used to calculate ΔG and TΔS using the standard thermodynamic equation shown below

$$-RT\ln Ka = \Delta G = \Delta H - T\Delta S$$

where R = gas constant (1.99 cal.K-1.mol-1), T = temperature in Kelvin (298.15 K), ΔG = change in free enthalpy, ΔS = entropy of binding.

II.4.22 Cleavage assay of Syndecan-1 recombinant protein

Cleavage assay for Syd-1 was led as previously described [68]. Briefly, recombinant syndecan-1 fusion protein was incubated with recombinant M60-like domain and plasmin at an enzyme/substrate ratio of 1:10 in 50ul of TNC (150mM NaCl, 10mM CaCl2, 50mM Tris/HCl, ph7.5 and 0.05% Brij35) buffer at 37°C for 4 hours. 50mM of metal chelator Ethylenediaminetetraacetic acid (EDTA), was also added to similarly prepared samples to test the metal dependency of recombinant proteins with cleavage activity. The digests were diluted with standard SDS concentration used for electrophoresis, boiled for 5 min and separated be electrophoresis on 12% SDS-PAGE. Membranes were stained for 10min in Comassie Brilliant Blue R-250.

II.4.23 Bioinformatic tools

MEROPS: database with information resources for peptidases and the proteins that inhibit them. (http://www.merops.sanger.ac.uk/)

Multalign: Multiple sequence alignment tools. (http://multalin.toulouse.inra.fr/multalin/)

ProtParam tool: Estimation of protein molecular weight and extinction coefficients. (http://web.expasy.org/protparam/)

Pfam 27.0: database with large collection of protein families, each represented by multiple sequence alignments and hidden Markov models (HMMs). (http://pfam.xfam.org/)

InterPro: provides functional analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites. (http://www.ebi.ac.uk/interpro/)

II.5 Results

II.5.1 Gene cloning and expression of domains from TVAG339720

For cloning and expression of three different gene fragments from TVAG339720 was used the gene synthesis pMUT318, encoding the extracellular domain of TvM60L zinc metallopeptidase under investigation.
M60-like domain, PA14-GBDL domains and GBDL domain encoding regions were generated by PCR using for each one a flanking forward primer with an extra BamHI site and a flanking reverse primer with an extra XhoI site. The oligonucleotide primers used in this study were listed in Appendix 1. The amplified DNA fragments were digested with BamHI and XhoI and cloned into pET28.a plasmid. The cloning strategy was planned to allow the introduction of an N-poly-histidine tag (His6) in each recombinant protein following expression.
The *E. coli* Tuner (DE3) expression host was used for the expression of cloned genes and the culture volume used was 1L or 2L. In all cases, the protein expression was induced with 0.1mM IPTG overnight at 16°C. Cells were collected and then sonicated in Talon 1x to improve the solubility of the expressed protein.
Recombinant N-His tagged proteins were purified using immobilized metal affinity chromatography (IMAC) (Section II.4.17). The concentration of purified proteins was evaluated by absorbance (A280nm) using the estimated molecular weight and extinction coefficient of each purified recombinant protein (Appendix B). All protein expression data are shown in the figure II.6.

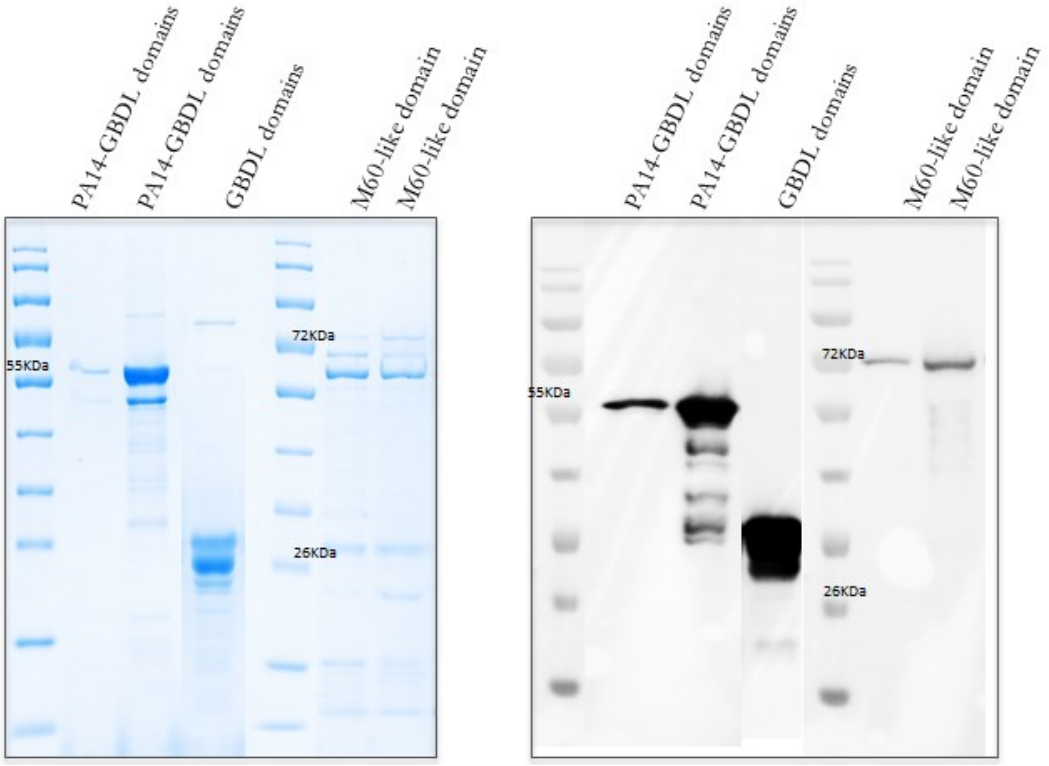


Figure II.6 - Expression of domains from TVAG339720

The expression of recombinant TvM60-like, TvPA14-GBDL and TvGBDL domains in pET28.a was evaluated by Comassie and Western blotting. Each protein was purified in 5ml of Talon 1x added with different concentration of imidazole. For analysis in SDS-page was loaded 5µl from total expressed proteins of TvPA14-GBDL and TvGBDL and 10µl from total expressed proteins of TvM60L. The expected sizes were 72.1kDa for TvM60L; 53.5kDa for TvPA14-GBDL, and 26.6kDa for TvGBDL. The antibody used to recognize expressed domains was anti-His-tag.

II.5.2 Carbohydrate binding modules of TVAG339720 M60like proteins.

The carbohydrate binding properties of PA14-GBDL domains and GBDL domain identified in TvM60like protein were studied by Isothermal titration calorimetry (ITC, Section II.4.21). The amount of proteins for ITC was obtained by ~1L of Tuner (DE3) culture. Both recombinant proteins revealed affinity for heparin and heparan sulphate. TvPA14-GBDL showed higher affinity for highly

sulphated GAG, heparin (Figure II.7) (Table II.9). The lower affinity for HS may be explained for the small amount of substrate used in these experiments, causing by expensive cost of heparan sulphate.

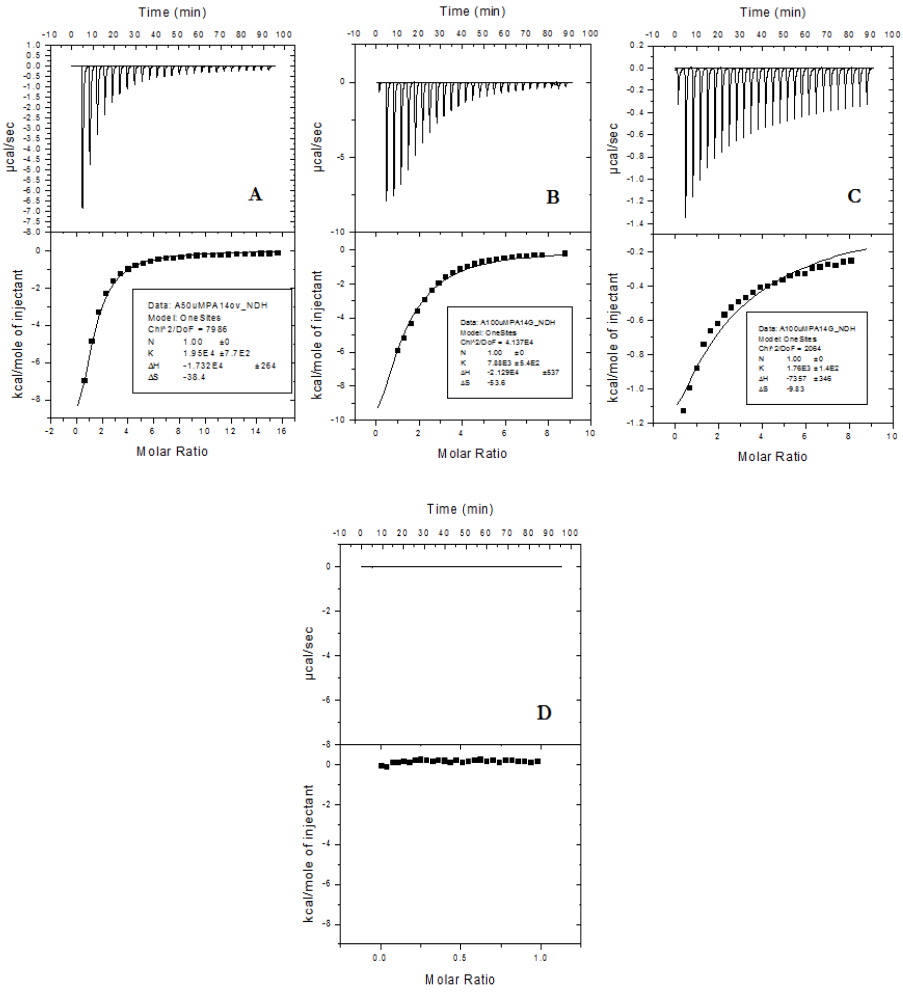


Figure II.7 - ITC data of TvPA14-GBDL binding to heparin and heparan sulphate.

For these experiments, the purified recombinant proteins were dialysed overnight into 50mM Sodium phosphate buffer pH 7. Samples were filtered the next day alongside ligands (heparin and heparan sulphate) that had been prepared in filtered dialyses buffer. The sugar in the syringe was titrated (27 injections) into the cell containing the protein sample and thermodynamic data analysed using Origin version 7.0 tool. A. 50µM TvPA14 vs 0.5% Heparin (Data from Dr. Ndeh thesis). B. 100µM TvPA14-GBDL vs 0.5% Heparin. C 100µM TvPA14-GBDL vs 0.5% Heparan sulphate. D. without sample (control).

Table II.9 - Affinity and thermodynamic parameters of TvPA14 and TvPA14-GBDL binding to heparin and heparan sulphate.

Thermodynamic parameters were calculated using the standards thermodynamic equation -RTlnKa = ΔG = ΔH- TΔS, where R = gas constant (1.99Kcal -1moli^{-1}), T = temperature in Kelvin (298.15 K), ΔG = change in free enthalpy, ΔS = entropy of binding, n = stoichiometry of binding.

Substrate	***Ligand***	***Ka × 10^3*** **(M^{-1})**	***ΔG*** **(kcal mol^{-1})**	***ΔH*** **(kcal mol^{-1})**	***TΔS*** **(kcal mol^{-1})**	**n**
Heparin	TvPA14	19.5 ± 7.72e^2	-5.88	-1.732 ± 264	-11.44	1
Heparin	TvPA14-GBDL	7.88 ± 537	-5.31	-21.29 ± 537	-15.98	1
Heparan sulphate	TvPA14-GBDL	-1.76 ± 1.4e^2	-7354	-7357 ± 346	-2.79	1

II.5.3 Cleavage assay of Syndecan-1

Cleavage assay of Syd-1 was performed to investigate the potential protease activity of expressed TvM60L domain. Human recombinant Syd-1 was incubated with recombinant TvM60L for 4 hours. The serine protease plasmin was included as positive control and EDTA incubated with TvM60L and Syd-1 was used to inhibit the protease activity of domain.

After enzyme treatment and separation by SDS-PAGE, there was no evidence of cleavage activity of TvM60L domain on recombinant Syd-1 *in vitro* (Figure II.8)

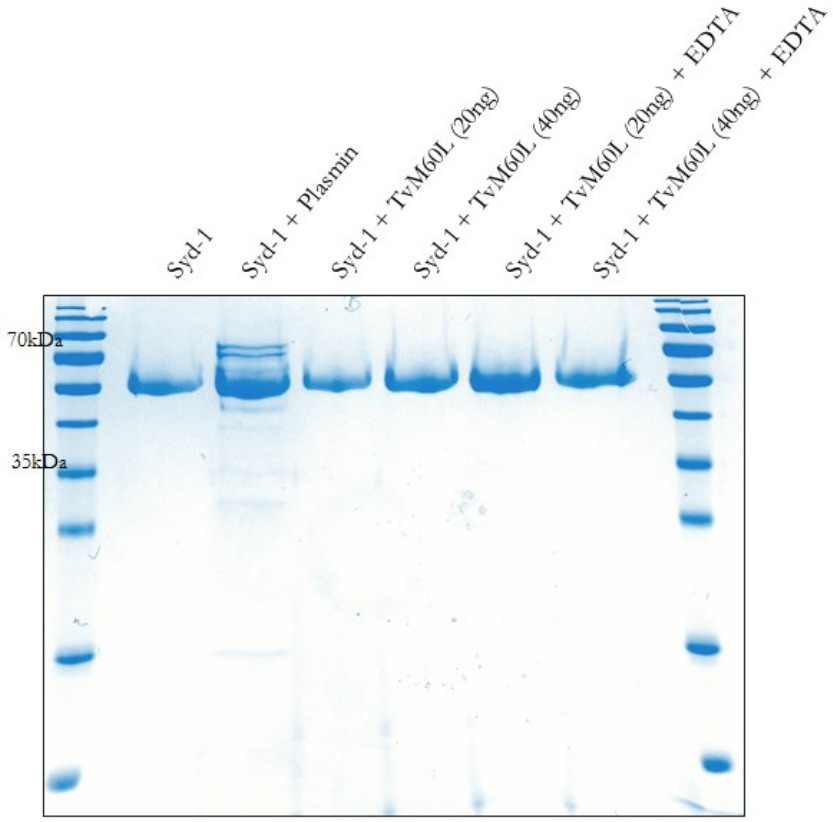


Figure II.8 Cleavage assay of human recombinant Syd-1.

Syd-1 was incubated with or without enzymes in a substrate/enzymes ratio of 1:10 in a total volume of 50µl of TNC buffer for 4 h, at 37°C and analysed on 12% SDS-PAGE. After electrophoresis gel was stained with Comassie Brillian Blue. The figure shows that human recombinant Syd-1 is not cleaved by recombinant TvM60L domain, *in vitro*.

CHAPTER III

Trichomonas vaginalis and *Mycoplasma hominis*: a mutualistic endosymbiotic relationship

III.1 Introduction

The interaction between *T.vaginalis* and *M.hominis* is the only endosymbiosis described so far involving two obligated human parasites that produce independent diseases in the same human region. However, the exact nature and fundamental aspects of this symbiosis still have to be clarified.

In the last few years several aspects of this symbiosis have been characterized. *M.hominis* has the capability to of entering and replicating in *T.vaginalis* (Figure III.1) [87]. The number of *M.hominis* organisms carried by *T.vaginalis in vitro*, varied from isolate to isolate, suggesting a specific multiplicity of infection [88]. Moreover, mycoplasmal infection can be passed from trichomonads to mycoplasma-free trichomonad isolates and to human cervical cells *in vitro* [88]. These data suggest that the intracellular location of *M.hominis* in trichomonads cells protects bacteria from the hostile environment and host immune response and *T.vaginalis* could play a role of "Trojan horse" for the bacterium during infection in the human body [56].

The presence of *M.hominis* in trichomonads induces changing in *T.vaginalis* cytopathogenicity *in vitro* [89]. Protozoa infected with mycoplasma displayed a higher amoeboid transformation rates and a more intense phagocityc capacity, then mycoplasma-free *T.vaginalis*. Moreover, hVECs coinfected with *T.vaginalis* associated with *M.hominis* show a decrease of viability and an increase in hVECs intracellular spaces and complete epithelial destruction [89].

Furthermore, we have recently demonstrated that the stimulation of the human monocytic cell line THP-1 with *T.vaginalis*, stably associated with *M.hominis*, synergistically upregulates the secretion of proinflammatory cytokines [90]. These findings suggest an influence of this relationship on the pathobiology of *T.vaginalis* too.

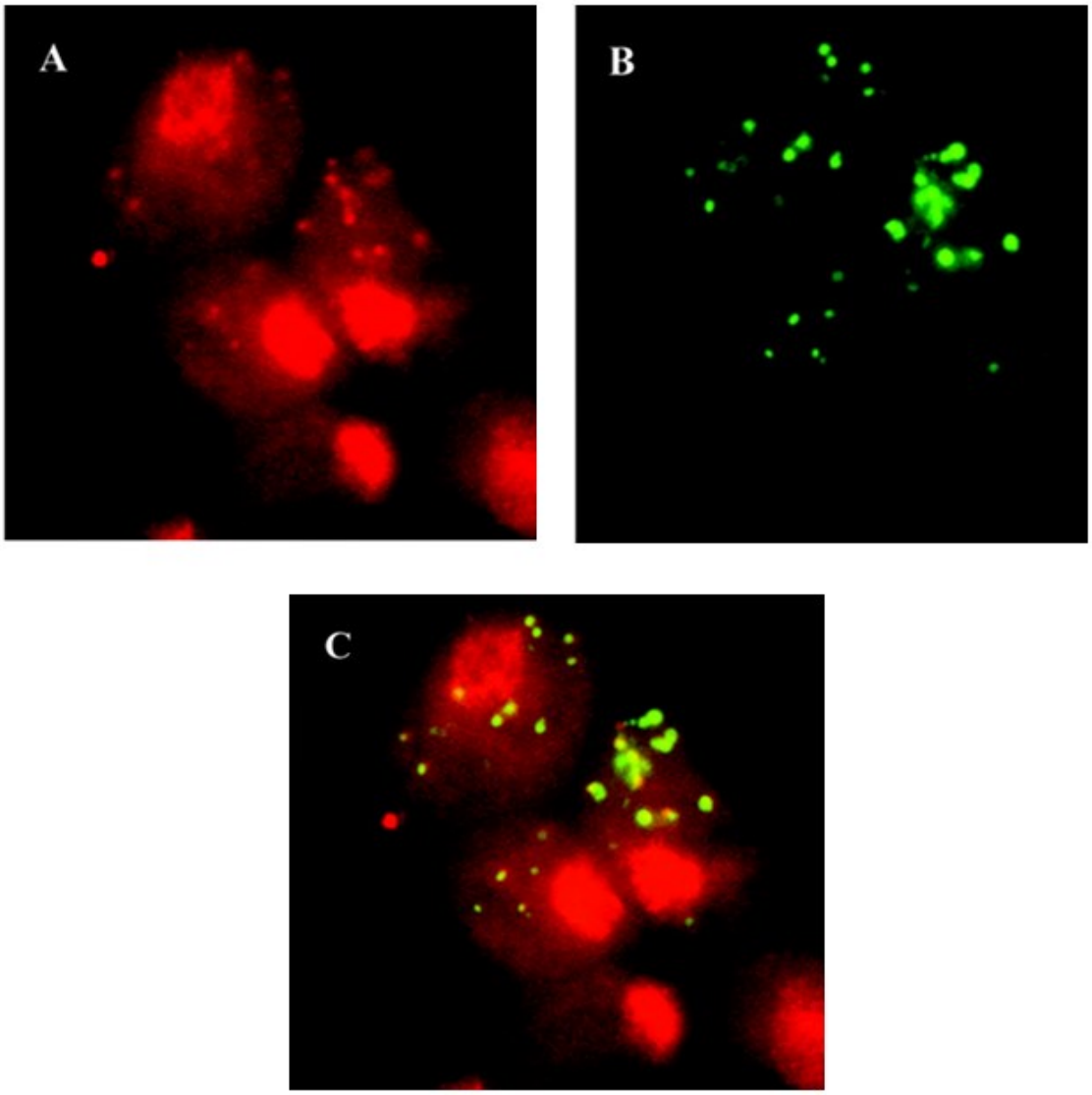


Figure III.1 - Intracellular localization of *M.hominis* within *T.vaginalis.*

The micrographs show the localization of *M.hominis* within *T.vaginalis* by detection of 5-BrdU incorporated in *M.hominis*. Panels show the same area of a protozoan monolayer. A. Incorporation of 5-BrdU (red spots) in *M.hominis* associated with *T.vaginalis*, detected by specific antibody. B. The same field showing *T.vaginalis* in symbiosis with *M.hominis* detected by anti *M.hominis* antibody. C. Overlapping of images A and B indicative of the exact colocalization of the two fluorescent signals. (Images modified from Dessì et al, 2005).

III.2 *Mycoplasma hominis*

Mycoplasma hominis is a bacterium belonging to Class Mollicutes, which resides as a commensal, in the lower urogenital tract of human beings (Table III.1). However, it can act as pathogen, causing pelvic inflammatory disease and postpartum or postabortion fevers, and has been associated with bacterial vaginosis. *M.hominis* is also implicated in extragenital infections in immunecompromised patients [91].

Table III.1 - Classification of *M.hominis.*

Domain:	Prokarya
Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Mollicutes
Order	Mycoplasmatales
Family:	Mycoplasmataceae
Genus:	Mycoplasma
Species:	*Mycoplasma hominis*

This bacterium shares the same natural niche, the urogenital tract, with *M.genitalium* and *Ureaplasma urealyticum*, having pathogenic roles in humans. Mycoplasma and Ureoplasma genera are collectively referred to as mycoplasmas. Mycoplasmas possess the smallest genome among self-replicating free living organisms, are often dependent on their hosts and lack cell walls [92].
The dominating shape is a sphere, albeit many Mollicutes exhibit a variety of morphological forms. The ability to maintain this form in absence of a rigid cell wall has indicated the presence of a cytoskeleton. Cytoskeleton is thought to participate in cell division, motility and the proper localization of adhesins. The reproduction is the binary fission, as other prokaryotes, but is still not clear the mechanism of this process.

The primary habitats of human mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, with organ and tissue specificity. These bacteria are able to enter in human cells. Some of them use a specialized tip structure to enter in the host cell and other mycoplasmas shown to internalize, such as *M.hominis*, have no tip structure. The intracellular location may protect mycoplasmas against the effects of the host immune system and antibiotics and allows the uptake of molecules which are incapable of synthesizing. In fact, the complete genome sequences of several Mollicutes revealed the lack of genes involved in amino acid and cofactor biosynthesis. The number of genes involved in lipid metabolism and purine and pyrimidine synthesis is extremely low, indicating that mycoplasmas are not able to synthetize fatty acids and must important nucleic acid precursor [92].

M.hominis can be considered as minimal bacterial prototypes with reduce metabolic abilities since its genome is composed by ~696Kbp. It's a nonglycolitic species, producing energy through arginine degradation [93].

III.3 Arginine dihydrolase (ADH) pathway: a common biochemical pathway

T.vaginalis and *M.hominis* show a common biochemical pathway, (ADH) [94]: in both organisms arginine is converted to ornithine and ammonia through the enzymes arginine deiminase (ADI), catabolic ornithine carbamyltransferase (cOCT) and carbamate kinase (CK), and finally ATP is generated by depletion of nitrogen from amino acids. In *T.vaginalis*, ADI are localized in hydrogenosomes, an anaerobic form of mitochondria, while the other enzymes of ADH localize to the cytosol (Figure III.2) [95] For *M.hominis* this is the major energetic pathway [93], while *T.vaginalis* uses it to acquire up to the 10% of its energy requirements [94]. Recently Huang K-Y et al have shown that OCT and CK are upregulated in the log growth phase of *T.vaginalis* grown in glucose-restriction (GR) on metabolism, suggesting that this energy –producing pathway is important for cell division under GR [96].

Interestingly, trichomonas cultures infected with *M.hominis* exhibited an increase in arginine consumption and in ornithine and putrescine production compared

to mycoplasma- free *T.vaginalis* [97]. These data suggest that an additional benefit for *M.hominis* may be the possibility to uptake molecules which is incapable of synthesizing, like putrescine. For *T.vaginalis* the advantage is debatable but, and given that *T.vaginalis* in symbiosis with *M.hominis* consumes larger amounts of free arginine compared to trichomonads alone, this metabolic association could contribute to down-regulate host innate defences. In fact, depletion of free arginine reduces nitric oxide (NO) production by macrophages and interferes with an important host defence mechanism [97].

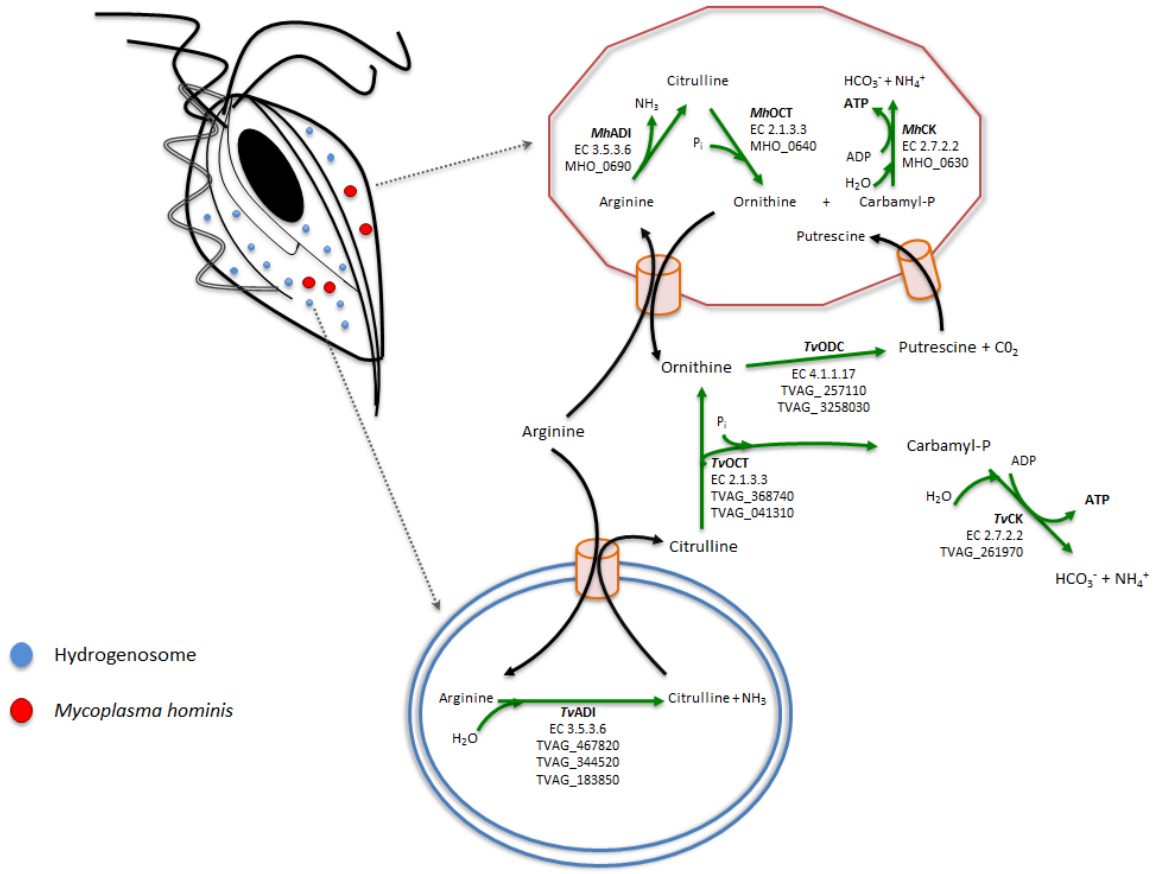


Figure III.2 - Arginine dihydrolase pathway in *T.vaginalis* and *M.hominis*.

Arginine is hydrolytically cleaved to citrulline by arginine deaminase (ADI, EC 3.5.3.6), citrulline undergoes phosporolysis by catabolic ornithine carbamiltransferase (OCT, EC 2.1.3.3) to ornithine and carbamyl phosphate. Ornithine is converted in putrescine by ornithine decarboxylase (ODC, EC 4.1.1.17) and carbamyl phosphate is broken down by a catabolic carbamate kinase enzyme (CK, EC 2.7.2) to bicarbonate and ammonia with the

concomitant phosphorylation of a molecule of ADP to ATP. By KEGG functional annotation, the enzymes involved in ADH both in *T.vaginalis* and in *M.hominis* have been reported

III.3 Macrophages and nitric oxide: an important host defence against *T.vaginalis*

Natural cell-mediated cytotoxicity against *T.vaginalis* is mediated by macrophages *in vitro* and their NO plays an important role in host defence against trichomonads cells [98, 99].

NO is one of the most widespread signalling molecules in mammals. It is implicated in modulating a variety of physiological reactions, including vasodilatation and smooth muscle relaxation associated with the circulation, functioning as a neurotransmitter in several neural processes, and regulation of immunological defence mechanisms [100]. The antimicrobial effects of NO are documented in a number of infection diseases, including intracellular (malaria, toxoplasmosis and leishmaniasis) and extracellular (giardiasis, cryptosporidiosis and trichomoniasis) parasitosis [101]. NO also plays a key role in the mechanisms of innate immunity given that it allows natural killer cells to respond to interleukin -12 and IFN-α and IFN-β stimuli at the onset of infection [102]. NO is produced by a nitric oxide synthases, a group of enzymes that convert arginine into citrulline, producing NO in the process. O2 and NADPH are necessary co-factors (Figure III.3). There are three isoforms of nitric oxide synthase (NOS): neuronal or nNOS or NOS1; endothelial or eNOS or NOS3 and inducible or iNOS or NOS2. nNOS and eNOS are constitutively expressed in mammalian cells and NO synthesis is linked to the increment in intracellular calcium levels. By contrast, iNOS activity is independent of the level of calcium in the cell and its expression in macrophages is controlled by cytokines and microbial products. Other macrophage products, such as acid, glutathione, cysteine, hydrogen peroxide or superoxide, enhance the antimicrobial and cytotoxic actions of NO [103].

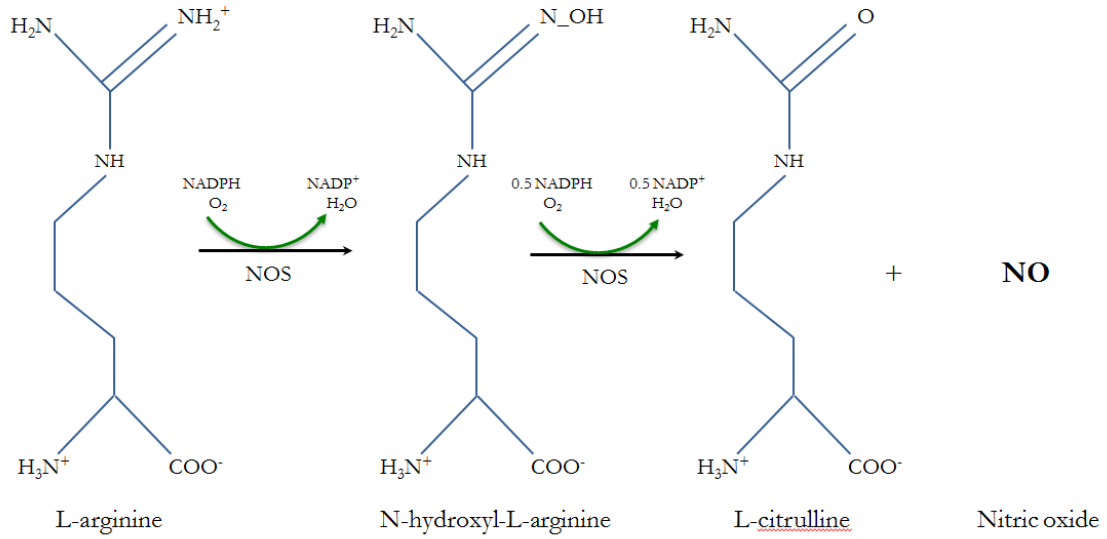


Figure III.3 - Biochemical pathway of NO production in mammalian cells.

NO biosynthesis consists of two sequential reactions catalysed by NO synthases. NOS in the presence of cofactors, as NADPH and oxygen, converts arginine into citrulline and NO.

III.4 Objectives

The purpose of this chapter is to shed light on some aspects of this symbiosis, initially studying the influence of *M.hominis* on production of ATP, by comparing Mycoplasma free *T.vaginalis* and protozoa stably associated with *M.hominis*, at different growth phases and arginine concentrations.

Then, investigating how the association between *T.vaginalis* and *M.hominis* may influence NO production by human monocytic cell line THP-1, in presence of different free arginine environmental concentrations, to understand likely competitive patterns.

III.5 Materials and methods

III.5.1 Materials and reagents

Cell culture media, RPMI 1640 with or without L-Arginine were obtained from GIBCO and Sigma-Aldrich. Lipopolysaccharides from *Escherichia coli* 055:B5, recombinant human INF-γ produced in *E.coli* and the Griess reagent were purchased from Sigma-Aldrich. Phorbol-12-myristate -13- acetate (PMA) was obtained from Sigma-Aldrich and fetal bovine serum (FBS) was purchased from GIBCO.

III.5.2 Parasites and culture condition

T.vaginalis strain G3, naturally mycoplasma- free, and experimentally infected with *M.hominis* isolate (MPM02) were cultured by daily passages at 1:16 in Diamond's medium (MDM) supplemented with 10% FBS at 37°C in a 5% CO2 atmosphere.

M.hominis cells were isolated from *T.vaginalis* as previously described [88], maintained in BEA medium (2.2% heart infusion broth; 15% horse serum; 1.9% yeast extract; 40 IU/ml benzylpenicillin; 0.23% L-arginine; 0.0023% phenol red, pH 7.2) and named by the originating trichomonad host.

In order to obtain an isogenic mycoplasma-infected trichomonad strain, *T.vaginalis* G3 was stably infected with *M.hominis* isolated from protozoa as previously described [97]. Briefly, 1 ml of an overnight culture of *M.hominis* isolates, corresponding to approximately 109 colour-changing units (CCUs), was added daily to a 10-ml mid-log phase culture of the *M.hominis*-free *T.vaginalis* isolate G3 for 5 days. Parasites were subsequently cultivated for 10 days with 1:16 daily passages. Stable *M.hominis* infection was then assessed by PCR with specific primers [104] and by isolating *M.hominis* in liquid and solid BEA medium. Multiplicity of Infection (MOI) was evaluated by plating bacteria from cell lysates in BE agar; plates were incubated for 3 days at 37°C in order to obtain colony forming unit. Cell cultures contaminated by mycoplasma and

mycoplasma free were grown in two different incubators in order to avoid cross contamination.

III.5.3 Cell culture and THP-1 differentiation

Human THP-1 monocytes leukaemia cells were cultured *in vitro* in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO2 and maintained at 5x105 cells/ml.
THP-1 cells were harvested at the exponential growth, seeded at 5x105 cells per well in 24-well plates and differentiated in macrophage (dTHP-1) by stimulation with 50ng/ml PMA for 18hours. Differentiation of PMA treated cells was enhanced by removing the PMA-containing media and incubating the cells in RPMI 1640 with or without L-Arginine, supplemented with 10% FBS for further 5 days.

III.5.4 Determination in vitro amount of intracellular ATP

The amount of intracellular ATP produced by *T.vaginalis* (Tv) and *T.vaginalis* infected with *M.hominis* (Tv-MhMPM02) was evaluated in three different phases of growth: lag, exponential, and stationary phase. Furthermore, it was analysed the concentration of ATP produced by microorganisms grown in media added to 0.1mM L-Arginine and 0.5mM L-Arginine. 15,000 cells of all samples were harvested and centrifuged at 4000 X g for 10 min and analysed in 50µl of medium through the kit CellTiter-Glo Luminescent Cell Assay (Promega).

III.5.5 Determination of NO produced by THP-1 co-incubated with infected and not infected *T.vaginalis*

To evaluate the release of NO by dTHP-1 in co-culture with parasites in vitro, *T.vaginalis* and *T.vaginalis* infected with *M.hominis* were harvested at the exponential growth and suspended in media with different concentration of arginine. Microorganisms were added in 24-weel plates containing differentiated adherent macrophages, at two different Tv/macrophages ratio, 1:5 to 1:10, in

presence of 100 Units/ml INF-γ. Untreated dTHP-1 and dTHP-1 stimulated with 1μg/ml of LPS were used as negative and positive controls respectively. The cell cultures were maintained at 37°C under a humidified 5% CO2 atmosphere and culture medium was harvested at 24 hours after infection.
NO produced by macrophage was determined measuring the nitrite levels in the culture medium through the Griess reaction. Briefly, culture medium was removed, centrifuged at 400g for 10 min and nitrite levels measured in supernatants. Equal volume of supernatants and Griess reagent (containing 0.1% naphthylenedamine dihydrochloride, 1% sulphanilamide and 2.5% H3PO4) was incubated for 15 min at room temperature in the dark. The absorbance was measured at 540nm in an ELISA reader. Nitrite concentration (μM) was determined using NaNO2 as standard.

III.5.6 Statistical analysis

All experiments were carried out at least in triplicate. We performed statistical analysis using the unpaired Student t test (Microsoft Excel; Microsoft, Redmond, Washington, USA). A p value <0.05 was considered significant.

III.5.7 Bioinformatic tools

KEGG: collection of databases about genomes, biological pathways, diseases, drugs, and chemical substances. (http://www.genome.jp/kegg/)

III.6 Results

III.6.1 *T.vaginalis* in symbiosis with *M.hominis* produces more ATP

Since both microorganisms utilize arginine as additional energy source via ADH pathway [97], and *M.hominis* utilizes the same metabolic pathway as unique ATP source, it was investigated the possibility that *M.hominis* may influence the amount of ATP produced by *T.vaginalis*. As observed in figure III.4, the ATP produced by Tv-MhMPM02 is higher than that produced by *T.vaginalis* alone in all growth phases.

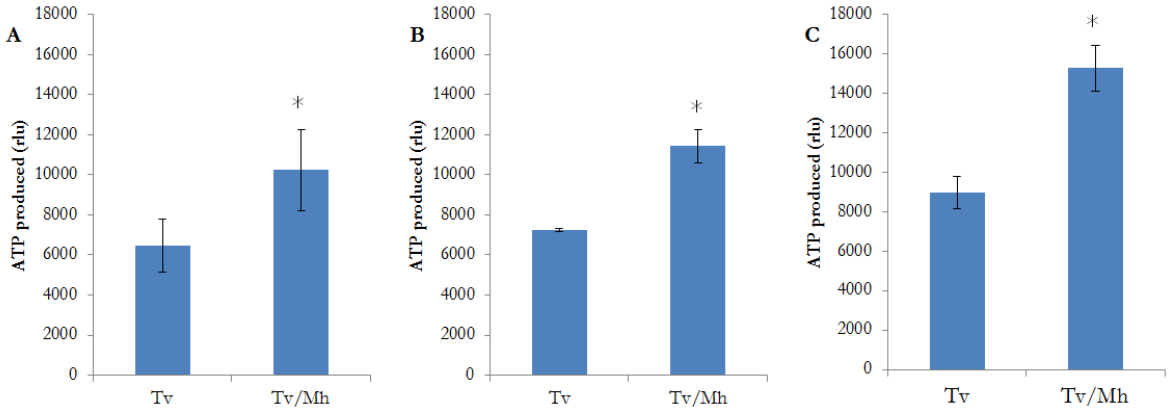


Figure III.4 - Intracellular concentration of ATP produced by *T.vaginalis* and *T.vaginalis* infected with *M.hominis* in different growth phases.

(A: lag phase; B: exponential phase; C: stationary phase). The amount of ATP produced by Tv-MPM02 is higher than that produced by Tv alone in all growth phases. Bars represent the mean ±S.D. of at least three independent experiments. Data were analysed by Student's t test. *p< 0.01 when intracellular concentration of ATP produced by *T.vaginalis*-MPM02 is compared to amount of ATP produced by mycoplasma-free *T.vaginalis*.

These data have led to examine whether arginine concentration in medium can influence ATP production by both infected and uninfected *T.vaginalis*. The later experiments were achieved adding 0,1mM and 0,5mM L-Arginine in medium. The ATP produced by Tv grown in medium supplemented of arginine is similar to G3 control cells growth in media not supplemented by arginine. Interestingly, in these experimental conditions, parasitized isolates are able to produce higher ATP amount compared to mycoplasma free *T.vaginalis* (Figure III.5).

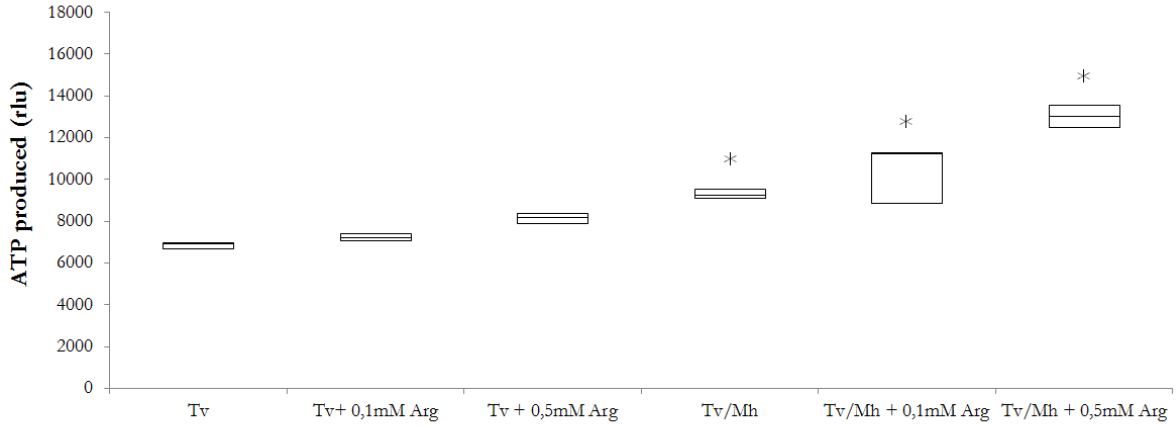


Figure III.5 - ATP produced by *T.vaginalis* G3 and *T.vaginalis* G3-MPM02 growth in media with different concentration of arginine.

The microorganisms have been grown in media supplemented with different concentrations of arginine, compared with the same strains cultured in normal Diamond's medium. ATP amount was measured from microorganisms in exponential phase. *M.hominis* infected trichomonads cells leads to an approximately 2-fold increase in ATP production compared to *T.vaginalis* alone, both in normal Diamond's medium and in medium added with arginine. Analyses represent averages of three independent triplicate experiments. Data were analysed by R project. *$p < 0.05$ by Linear mixed-effects model fit by REML

These results demonstrate that the presence of *M.hominis* in trichomonads cells leads to an approximately 2-fold increase in ATP production, compared to mycoplasma-free *T.vaginalis* cultures, both in normal and in arginine supplemented media

III.6.2 *M.hominis*-infected *T.vaginalis* competes with host macrophages for arginine and prevents NO formation

Depletion of host arginine could be considered as a microbial virulence strategy preventing toxic NO formation by macrophages. Since it was previously demonstrated that *M.hominis* infected protozoa are able to produce larger amount of ADI compared with Mycoplasma-free isolates [97], it was evaluated NO production in human macrophages stimulated with *T.vaginalis*-infected and not infected by *M.hominis*.

THP-1 cells were differentiated to a macrophage-like phenotype by PMA (dTHP-1) and were thereafter infected with both *M.hominis*-free and *M.hominis* -infected *T.vaginalis*. The experiments have been led using two different MOI (5:1 and 10:1) (macrophages vs. protozoa). Macrophages were stimulated with INF–γ at time of infection. *E.coli* LPS was used as positive control, while unstimulated dTHP-1 were used as negative control. The supernatants were harvested after 6h and 24h from microbial stimulation and it was evaluated the amount of nitric oxide. After 6 hours from infection there was not considerable difference in production of nitric oxide among macrophages infected with G3 and G3-MPM02 (data not shown). In contrast after 24hours of incubation, we have observed higher secretion of NO by dTHP-1 in response to infection of G3-MPM02 compared to mycoplasma-free trichomonads (Figure III.6).

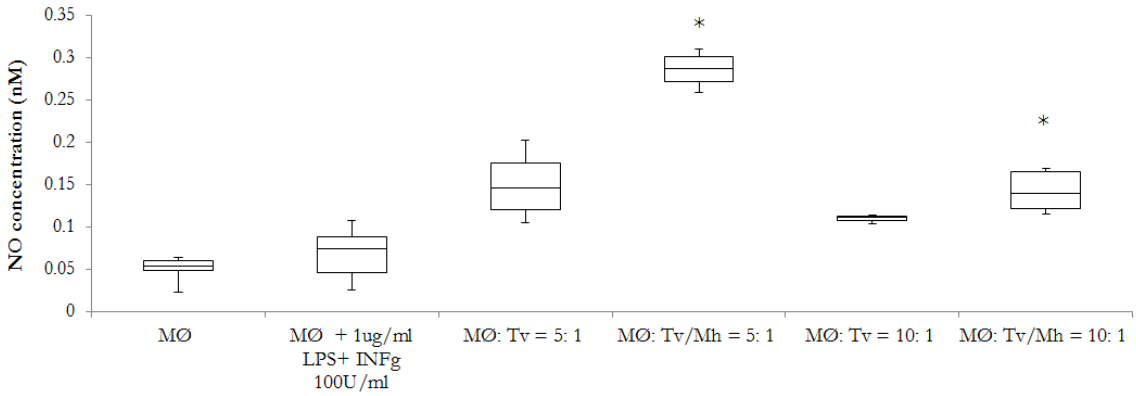


Figure III.6 - NO produced by macrophage after cocolture with pathogens.

Amount of NO produced by dTHP-1after 24 hours from infection with *T.vaginalis* G3 and *T.vaginalis* G3-MPM02. Experiments were carried out in the ratio 5:1 and 10:1 macrophages to protozoa. THP-1 cells grown in medium with arginine produce more amount of NO in response to infection with G3-MPM02 compared mycoplasma-free G3. The values represent averages of at least three independent experiments. Data were analysed by Student's t test.*p value<0.05 when amount of NO produced by dTHP-1 infected with G3-MPM02 is compared with amount of NO release by macrophages infected with G3 in all ratio. Macrophages unstimulated and stimulated with LPS and INFγ have been used respectively as negative and positive controls.

These data show that infected strains not only do not inhibit but also up-regulate NO production by macrophages. This apparently paradoxical result can be explained by assuming that trichomonads (infected or not infected) were not able to completely deplete arginine amounts normally present in culture media. In these experimental conditions, mycoplasma infecting *T.vaginalis* cells synergistically stimulates NO production from dTHP-1. In order to demonstrate if *M.hominis* arginine metabolism in infected trichomonads could contribute on subtraction of this amino acid and, by consequence, inhibit NO production by macrophages, THP-1 cells were differentiated and cultured in arginine-free media to deplete intracellular store. dTHP-1 were then stimulated with infected and not-infected *T.vaginalis* in presence of 0.1 and 0.5mM arginine, using ratio macrophages: pathogen 10:1, in order to avoid the cytotoxic effect of *T.vaginalis*

on macrophages. Interestingly, it was observed a decrease in production of nitric oxide by macrophages infected with *T.vaginalis* in symbiosis with *M.hominis* compared with NO derived from THP-1 stimulated with G3 alone, in presence of both 0.1 and 0.5mM arginine (Figure III.7).

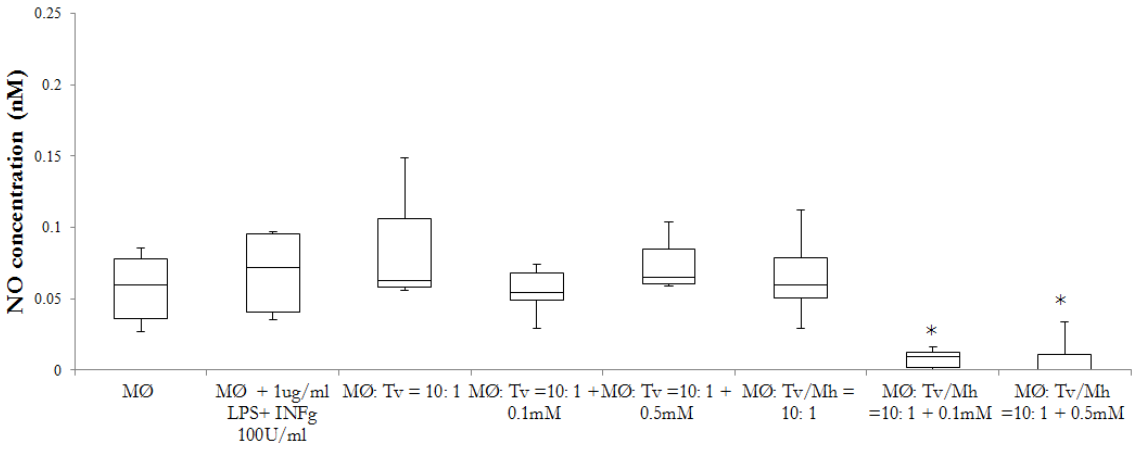


Figure III.7 NO produced by macrophages infected with pathogen in media with different arginine concentrations.

Amount of NO produced by dTHP after 24 hours incubation with *T.vaginalis* G3 and G3-MPM02 in medium added with 0.1 and 0.5mM arginine. Experiments were carried out in the ratio 10:1. There is a marked reduction of NO produced by dTHP-1 infected with G3-MPM02 while the amount of NO in supernatants from dTHP-1 infected with *T.vaginalis* is almost the same in all conditions. G3-MPM02 is able to capture more arginine then trichomonads alone, reducing NO production by macrophages. Analyzes represent averages of three independent triplicate experiments. Data were analyzed by R project. *$p<0.05$ by Linear mixed-effects model fit by REML

Results suggest that *T.vaginalis* and *M.hominis* in symbiosis are able to capture more arginine then trichomonads alone, reducing NO production by macrophages.

CHAPTER IV

Final discussion

Trichomonas vaginalis is the ancient protist causing trichomoniasis, common cosmopolitan non-viral sexually transmitted infection. Humans are the only natural host of *T.vaginalis* and despite the high prevalence and incidence of this disease, *T.vaginalis* is one of the most poorly studied parasites about virulence properties, pathogenesis and immunopathogenesis.

The pathobiology of protozoan is multifaceted and involves direct and indirect contacts with host tissue, bacteria, human viruses and *Trichomonas* viruses.

The sequencing of entire genome of *T.vaginalis* G3 has facilitated the study of the molecular basis of the parasite pathobiology.

In this work, we attempted to clarify some aspect of T.*vaginalis* pathogenesis, focusing studies on mechanisms involved in adhesion to host cells and on ability of protozoan to interact with human microbiota.

Data obtained from this study may contribute to better understand several mechanisms used by trichomonads cells to initiate the invasion of host mucosa and to sustain infections in the human host.

IV.1 TvM60-like protease involves in adhesion

T.vaginalis is an obligate extracellular parasite and adherence to epithelial cells is critical for parasite survival within human host [2] Surface proteins thus represent important elements in host-microbial interactions involving both pathogenic and mutualistic mucosal microbes.

Recently, Nakjang et al have discovered by comparative genomics analysis, a new family of proteins: M60-like domain containing proteins. The new domain, M60-like (PF13402), was detected mainly among proteins from animal host mucosa-associated microbes ranging from mutualists to pathogens [41]. The novel family is characterised by a zinc- metallopeptidase-like motif, by the presence of carbohydrate-binding modules and other glycan binding domains; and by the presence of SP and of transmembrane domain. These features suggesting that

these proteins target an extracellular substrate, as glycoproteins. A mucinase assay i*n vitro* has shown degradation of mammalian mucins by a recombinant form of M60-like proteases from the gut mutulist *Bacteroides thetaiotaomicron* [41].
In *T.vaginalis* were identified 25 M60-like domain-containing proteins and only three of them possessing a complete structure of M60-like proteins: TVAG199300, TVAG339720, and TVAG189150. The investigation for *T.vaginalis* was however problematic for the difficult in expressing in soluble form of these proteins in *E.coli*. A mucinase assay *in vitro* was performed for TVAG199300-M60L showing a failed to degrade mucins but the binding assay between PA14 domain of TVAG339720 and heparin suggested that these putative proteases may degrade proteoglycans that constitute the glycocalyx of mucosal epithelial cells (data from Dr.Didier Ndhe thesis).

In the I Chapter we attempted to characterize domains containing in TVAG339720 [41]. This protein is the only one shared by six tested isolates of *T.vaginalis*, possessing a complete M60-like domain. TvM60L, TvPA14-GBDL and TvGBDL domains were expressed in Tuner strain host in soluble forms and in significant amount, allowing functional studies.

PA14 domain is so named for the presence of this domain in the protective antigen (PA) region of the complex anthrax toxin. This domain is conserved and shared by a wide variety of bacterial and eukaryotic proteins, such as glycosidases, glycosyltransferases, proteases, amidases, adhesins, and bacterial toxins. PA14 domains and CBM32 have been detected in several M60-like proteins from several mucosal microbes, such as *Candida albicans*, to target galacto-configured sugars [86].
Moreover, PA14 sequence alignment and domain distribution suggest a carbohydrate binding function. These knowledge, along with data from other microbes producing PA14 domain, suggest that this domain may be involved in adhesion function via binding to sugar moieties of host glycolconjugates from glycocalyx, VEC, host immune cells and from ECM [105].

Previous study led by Dr.Didier Ndhe has shown that TVAG339720-PA14 binds to heparin with higher avidity compared with mucins, preferring highly sulphated heparin derivatives. These findings may be an indication that sulphate group is important in heparin recognition by the PA14 domain.
Heparin is structurally close to heparan sulphate. Both sugars are GAGs involved in epithelial cell glycocalyx, which can act both as protection of epithelial cells against pathogens and as receptor for microbial adhesion. Heparan sulphate chains are a common link that many pathogens, such as HSV, HIV, *C.trachomatis*, require for adhesion to eukaryotic cells, representing a primary mechanism during mucosal infection [60].
These data led us to hypnotize that the targets for M60-like PA14 domains are heparan sulphate glycosaminoglycans, which are very similar to heparin GAGs and take part to glycocalyx.
During PhD project, we have collaborated with Prof. Hirt and Dr. Ndhe of Newcastle University, studying the binding ability of TvPA14-GBDL and TvGBDL to heparin and heparan sulphate, to evaluate if there are difference in energy of binding between TvPA14 and TvPA14-GBDL and if TvGBDL is capable to bind to sugar.
Using ITC to study carbohydrate binding properties of *T.vaginalis* domains, we have discovered that the binding between heparin and TvPA14-GBDL is higher compared with TvPA14 alone and TvGBDL alone (data not show). Moreover, the reactions were exothermic and the saturation trend observed for the protein samples compared to the control without protein was suggestive of binding interaction. The next step was assessed the binding between TvPA14-GBDL and heparan sulphate. Also in this reaction, there was binding but less strong compared with energy obtained from TvPA14-GBDL with heparin, likely for small amount of sugar (HS) used for assay, caused by expensive cost of it.

Several studies have shown that various microbial pathogens activate the ectodomain shedding of several proteins from host cell surface to promote their pathogenesis and to dysregulate the host response [70]. Extracellular proteinases secreted by *Pseudomonas aeuroginosa*, *Enterococcus faecalis*, and *Streptococcus pyogenes*

shed dermatan sulphate proteoglycans, which bind to and inactivate neutrophil-derived α-defensin [106].

Proteoglycan along with glycoproteins, glycolipids and transmembrane mucins, constitute glycocalyx of human cells, which can act both as protection of epithelial cells against pathogens and as receptor for microbial adhesion.

The most common HSPG is represented by syndecan family of trans-membrane proteins. These include syndecan-1, -2, -3, and -4. HS chains of syndecans interact with several proteins, including growth factors, morphogens and extracellular matrix proteins, as well as with many bacteria, protozoa and viruses [59]. Syndecan-1 and syndecan-4 can mediate *N.gonorrhoeae* uptake into epithelial cells during syphilis infection, and *T.pallidum* binds to layer of GAGs by specific lipoproteins and coats itself with GAGs [60].

An important mechanism involves in microbial pathogenesis and host defence is the shedding of syndecan-1 ectodomain. The intact ectodomain of Syd-1 is shed from cultured cells as part of normal cell surface HSPG turnover and appears to contribute to several pathophysiological events such as host defense, arthritis and Alzheimer's disease [68]. Shedding of ectodomain involves the activity of cell surface zinc-metalloproteinases, e.g. MMP7, MMP9, MT1-MMP (MMP14) and ADAM 17, known to cleave ECM proteins in normal and pathological conditions [107]. Several pathogens, such as *P.aeruginosa*, *S.aureus* and *S.pneumoniae,* are also able to induce Syd-1 shedding, enhancing bacterial virulence by binding and inhibitions of various host defence factors, such as antimicrobial peptides, in HS dependent manner [71].

The novel family of domains termed "M60-like/PF13402" domains show distant relatives of viral-enhancin proteases known to degrade insect mucins [41] and were also found in *T.vaginalis* genome. The taxonomic distribution, domain content and sequence feature suggest that M60-like proteins could represent important surface zinc-metalloprotease enzymes processing extracellular glycoprotein targets.

Previous analyses have shown that genes of TVAG189150 and TVAG339720 encode for *T. vaginalis* M60-like domain-containing proteins reportedly express on cell surface [38]. Furthermore, our collaborators in the USA have confirmed the extracellular localization of the TVAG339720 protein by

immunofluorescence assay, showing that overexpression of protein in *T. vaginalis* itself increases vaginal epithelial cell cytolysis *in-vitro* (personal communication of Prof. Robert Hirt). Then, these observations led us to investigate *in vitro* the ability of M60-like domain of TVAG339720 to cleave proteoglycans. We performed a cleavage assay using as substrate human recombinant Syd-1, expressing HS chains. We have performed this assay with Syd-1 given that this HSPG is implicated in several microbial pathogenesis and is expressed in urogenital tract of both sexes [108, 109]. Moreover, Chen et al have shown an increase of expression of Syd-1 with more aggressive prostate cancers [109].
The recombinant TvM60L failed to cleave recombinant Syd-1 *in vitro*, likely because other parts of protease, such as PA14 and GBDL domains, may be important for identification, binding and then cleavage of proteoglycans.

Although we obtained negative result in the cleavage assay and it is not clear yet which are the targets for *T.vaginalis* M60-like proteases, the hypothesis that TVAG339720 and its close relatives may represent important virulence factors for *T.vaginalis* is still supported by binding between CBMs and HS-GAG. *T.vaginalis* could use these proteases against proteoglycan structures of glycocalyx for adhesion of mucosal epithelial cells, increasing number of proteins involved in the initial attachment to host tissue.
Moreover, the association of carbohydrate binding modules with proteases also represents a novel functional context interesting for further studies.

IV.2 Influence of *M.hominis* on pathobiology of *T.vaginalis*

The ability of *T.vaginalis* to interact with human microbiota, human pathogens and TVV present in the urogenital tract, is a mechanism involves in colonization of host mucosa and in recruitment of energy sources.
By phagocytosis, the pathogen is able to internalize bacteria and fungal cells of human microbiota, inducing dysbiosis observed during bacterial vaginosis [48]. Also, the ingestion of different human cells, as epithelial cells, immunocytes and spermatozoids, allow the pathogen to acquire nutrient and to defence from immune system. Moreover, the interaction between *T.vaginalis* with host

microbiota is confirmed by acquisition of a number of bacterial genes through lateral gene transfer (LTG). Interestingly, a set of enzymes of bacterial origins capable of degrading glycans are found in *T.vaginalis* proteome [110]. Mostly of these enzymes have been acquired from members of bacteroidetes, the most abundant bacteria lineage of mucosal microbiota [34]. These *T.vaginalis* enzymes may play an important role in colonization of host mucosal surfaces, helping in the adhesion to epithelial cells, by degradation of component of glycocalyx, such as mucins and other glycans. Furthermore, glycan degradation is thought to provide an important energy source for the pathogen.

T.vaginalis is also able to internalize virus, such as HIV and four human viruses members of Totiviridae, by endocytosis. The physically interaction with HIV facilitates the virus transmission through damaging mucosal surfaces and increases its replication [16]. Recent study has shown that the presence of TVV in the pathogen induces an innate response, including a proinflammatory cascades [47]. These considerations clearly illustrate the importance of interaction with human microbes in the pathogenesis of *T.vaginalis*.

In particular, the object of study in the III Chapter was the symbiosis that *T.vaginalis* establishes with an opportunistic human pathogen, *Mycoplasma hominis*. This relationship it is the first described between two microorganisms capable of independently inducing diseases in the same human region, the lower urogenital tract, and at the present, it is not still able to assess whether *M.hominis* actively invades *T.vaginalis* or is phagocytized by protozoan.

Clinical studies have shown a closer correlation between *T.vaginalis* and *M.hominis*. In fact, the bacterium is detectable in 79% of patients affected by trichomoniasis [111].

Studies *in vitro* have elucidated some aspects of this association as the ability of *M.hominis* to invade, survive and multiply in the *T.vaginalis* cytoplasm [112, 113]. The capability of mycoplasma to enter *T.vaginalis* cells may represent an important defence mechanism for bacteria during human infection, so much to consider protozoa as Trojan horse in mycoplasma infections [114]. It has also been shown as *T.vaginalis* in symbiosis with *M.hominis* up regulates the secretion of proinflammatory cytokines by macrophage [90]. Furthermore, *T.vaginalis*

associated with mycoplasma presents a higher amoeboid transformation rate and an intense phagocytic activity, characteristic of higher virulence behaviour [89]. Although this interaction may have a considerable clinical impact, so far the knowledge about its nature are few.

In this chapter we attempted to clarify some aspects of this symbiosis, focusing our study on how *M.hominis* may influence the pathobiology of *T.vaginalis*.

To better understand if the presence of *M.hominis* confers advantages *T.vaginalis* cultures we have studied the arginine dihydrolase (ADH), a biochemical pathway shared by both microorganisms. While in trichomonads this pathway contributes about 10% of the total energy requirements in anaerobic condition [94, 115], in *M.hominis* is the major energetic pathway [93] and previous study have shown as mycoplasma benefit from this symbiosis through a constant supply of putrescine from cytosol of T.vaginalis [97], given that *M.hominis* is incapable of synthesizing it.

Since *T.vaginalis* infected with *M.hominis* contains two different ADH pathways (protozoan and bacterial origin) and that these pathways remove nitrogen from amino acids with the generation of ATP, we have investigated the hypotheses that *M.hominis* may influence the amount of ATP produced by *T.vaginalis*, comparing ATP generated by *T.vaginalis* alone and *T.vaginalis* stably associated with *M.hominis*. Experiments have been performed culturing pathogens in media with different concentration of arginine and the amount of ATP produced by *T.vaginalis* and *T.vaginalis* stably associated with *M.hominis* was obtained from cells in different growth phases. Our results demonstrate that the amount of ATP produced by Mycoplasma-infected *T.vaginalis* was higher than that produced by uninfected *T.vaginalis*, both in different growth phases and at different arginine concentrations. Given that previous data demonstrated that the presence of intracellular *M.hominis* do not influence the expression of ADI genes [97], we can hypothesize that the higher amount of ATP in infected strains is a consequence of the activity of bacterial and protozoan pathway.

These results lead us to speculate that *T.vaginalis* benefits from this symbiosis. The availability of this additional source of energy could be explained by the capacity of mycoplasmas to export ATP into the host cytoplasm [116], or ATP

could be available after partial or total digestion of bacteria by trichomonads [117, 118].

The paper of Morada et al, further demonstrated that *T.vaginalis* cultures infected with *M.hominis* increased free arginine consumption, a substrate for the hosts production of nitric oxide (NO) [97].

NO, a key effector of the immune system, is produced by NOS in several cell types, including macrophages, [119] and the antimicrobial effects of NO are documented in a number of infection diseases [101].

The role of NO in the pathogenesis of *T.vaginalis* is controversial. Some studies report that macrophage cytotoxicity against *T.vaginalis* is regulated by NO, as well as by proinflammatory cytokines, such as IL-1, IL-6 and TNFα via NF-kBl, and that macrophage –produced NO is toxic for *T.vaginalis* under aerobic conditions and [98, 99]. On the other hand, trichomoniasis often evolves in chronic infection, with the parasite able to survive in the microaerophilic vaginal environment NO-rich, escaping the hosts killing mechanisms. Moreover, Sarti et al have shown how *T.vaginalis* evades the NO-based host immune response, conferring an advantage for survival, by degradation of NO in efficiently way [120].

Given that the presence of *M.hominis* in *T.vaginalis* is essential for the robust increase of inflammatory mediators by macrophages [90] and that pathogens and immune system cells may compete for the same substrate, we have investigated if *M.hominis* in *T.vaginalis* influenced the synthesis of NO by phagocytic cells. After 24 hours incubation we have detected a higher secretion of NO in response to infection of infected *T.vaginalis*, compared to the amount of NO production in macrophages stimulated with uninfected protozoa. Due to the high arginine concentration in culture media, we think that mycoplasma pro-inflammatory molecules (such as membrane lipoproteins) could synergistically stimulate human macrophages. Interestingly when experiments were performed in media with low arginine, comparable with higher concentrations presents in commercial medium, we have observed a robust decrease in production of nitric oxide by macrophage stimulated with *T.vaginalis* associated with *M.hominis*. These results suggest a competition for acquiring of free arginine between protozoa and phagocytes. Furthermore, *M.hominis*-infected *T.vaginalis* able to capture

higher arginine compared whit uninfected trichomonads, strongly reducing nitric oxide production by macrophages.

Based upon the data obtained in this study, we conclude that the symbiosis between *T.vaginalis* and *M.hominis* is a mutually beneficial endosymbiotic relationship. Mycoplasma is able to influence both the biology and the immunopathology of *T.vaginalis in vitro*. Furthermore, the ability of *T.vaginalis* associated with *M.hominis* to uptake arginine, may indicate the capacity of microorganisms to acquire arginine from vaginal environmental *in vivo*, interfering and evading an important host defence mechanism. Moreover, *T.vaginalis* upregulating OCT and CK in the log growth phase of glucose restriction-cultivated cells, suggest that ADH pathway is important to maintain growth and survival in critical condition.

M.hominis might play a key role in inflammation during trichomoniasis, by modulating of *T.vaginalis* pathogenicity by upregulation of the macrophage proinflammatory response and by increasing of capacity of *T.vaginalis* to capture arginine from environment.

These data suggest a role of the symbiosis in the high degree of variability of signs and symptoms observed in *T.vaginalis* infection.

IV.3 Future works

T.vaginalis pathogenesis mechanisms analysed in this study illustrate the complex interactions taking place both between pathogen and host cells, and between protozoan and other human pathogens, such as *M.hominis*.

Future studies can be lead to better understand the mechanism involves in pathogenesis of *T.vaginalis* include

1) Recognition and characterization of amino acids implicate in HS-binding motifs present on TvPA14, TvPA14-GBDL and TvGBDL domains, assuming that binding to heparin and HS is dependent upon electrostatic interactions between positively charged regions of ligand and the negatively charged sulphate groups of the GAG receptor;
2) Screen other potential human proteoglycans in addition to syndecan-1 to evaluate protease activity.

3) Infection of VECs and prostate cancer cells with *T.vaginalis* overexpressing TvM60L-proteins, such as TVAG339870, to evaluate if there is shedding of proteoglycans from human cells, by recognition with specific antibody;

4) Investigation on role of *M.hominis* in *T.vaginalis* in progression of prostate cancer, in order to understand if this symbiosis is implicated also in tumorigenesis of prostate cancers;

5) Recognition, characterization of arginine transporters in *T.vaginalis* cell surface, and investigation on role of these transporters in survival and growth of *T.vaginalis*.

References

1. WHO: **Global incidence and prevalence of selected curable sexually transmitted infections-2008**. In: *World Health Organization*. Geneva, Switzerland; 2012: 1-20.

2. Petrin D, Delgaty K, Bhatt R, Garber G: **Clinical and microbiological aspects of Trichomonas vaginalis**. *Clinical Microbiology Reviews* 1998, **11**(2):300-317.

3. Yap EH, Ho TH, Chan YC, Thong TW, Ng GC, Mo LC, Singh M: **Serum antibodies to Trichomonas vaginalis in invasive cervical cancer patients**. *Genitourinary Medicine* 1995, **71**(6):402-404.

4. Hobbs M, Sena A, Swygard H, al e: **Trichomonas vaginalis and trichomoniasis**. In: *Sexually transmitted disease*. Edited by Holmes K, Holmes K, Sparling P, al e, 4th edn edn. New York: McGraw-Hill; 2008: 771–793.

5. Fichorova RN: **Impact of T. vaginalis infection on innate immune responses and reproductive outcome**. *Journal of Reproductive Immunology* 2009, **83**(1-2):185-189.

6. Sutcliffe S, Neace C, Magnuson NS, Reeves R, Alderete JF: **Trichomonosis, a Common Curable STI, and Prostate Carcinogenesis-A Proposed Molecular Mechanism**. *PLoS Pathogens* 2012, **8**(8).

7. Sutcliffe S, Giovannucci E, Alderete JF, Chang TH, Gaydos CA, Zenilman JM, De Marzo AM, Willett WC, Platz EA: **Plasma antibodies against Trichomonas vaginalis and subsequent risk of prostate cancer**. *Cancer Epidemiology Biomarkers and Prevention* 2006, **15**(5):939-945.

8. Twu O, Dessí D, Vu A, Mercer F, Stevens GC, De Miguel N, Rappelli P, Cocco AR, Clubb RT, Fiori PL *et al*: **Trichomonas vaginalis homolog of macrophage migration inhibitory factor induces prostate cell growth, invasiveness, and inflammatory responses**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**(22):8179-8184.

9. McClelland RS, Sangaré L, Hassan WM, Lavreys L, Mandaliya K, Kiarie J, Ndinya-Achola J, Jaoko W, Baeten JM: **Infection with Trichomonas vaginalis increases the risk of HIV-1 acquisition**. *Journal of Infectious Diseases* 2007, **195**(5):698-702.

10. Guenthner PC, Secor WE, Dezzutti CS: **Trichomonas vaginalis-induced epithelial monolayer disruption and human immunodeficiency virus type 1 (HIV-1) replication: Implications for the sexual transmission of HIV-1**. *Infection and Immunity* 2005, **73**(7):4155-4160.

11. Thurman AR, Doncel GF: **Innate Immunity and Inflammatory Response to Trichomonas vaginalis and Bacterial Vaginosis: Relationship to HIV Acquisition**. *American Journal of Reproductive Immunology* 2011, **65**(2):89-98.

12. Hirt RP dMN, Nakjang S, Dessi D, Liu YC, Diaz N, Rappelli P, Acosta-Serrano A, Fiori PL, Mottram JC.: **Trichomonas vaginalis pathobiology new insights from the genome sequence.** *Adv Parasitol* 2011, **77**:87-140.

13. Ryan CM dMN, Johnson PJ.: **Trichomonas vaginalis: current understanding of host-parasite interactions.** *Essays Biochem* 2011, **51**(161-75).

14. Harp DF CI: **Trichomoniasis: evaluation to execution.** *Eur J Obstet Gynecol Reprod Biol* 2011, **157**(1):3-9.

15. Pereira-Neves A RK, Benchimol M.: **Pseudocysts in trichomonads--new insights.** *Protist* 2003, **154**((3-4)):313-329.

16. Hobbs MM SA, Swygard H et al: ***Trichomonas vaginalis*** **and trichomoniasis** *In: Holmes K, Holmes KK, Sparling PF, et al eds Sexually transmitted disease* 2008, **4th edn, New York: McGraw-Hill**:771–793.

17. Dyall SD JP: **Origins of hydrogenosomes and mitochondria: evolution and organelle biogenesis.** *Curr Opin Microbiol* 2000, **3**((4)):404-411.

18. van der Giezen M SD, Horner DS, Dyal PL, Harding M, Xue GP, Embley TM, Kunji ER.: **Conserved properties of hydrogenosomal and mitochondrial ADP/ATP carriers: a common origin for both organelles.** *EMBO J* 2002, **21**((4)):572-579.

19. Muller M: **The hydrogenosome**. *Journal of General Microbiology* 1993, **139**((12)):2879-2889.

20. Embley TM vdGM, Horner DS, Dyal PL, Bell S, and Foster PG: **Hydrogenosomes, mitochondria and early eukaryotic evolution**. *IUBMB Life* 2003, **55**((7)):387-395.

21. Steinbüchel A aMM: **Glycerol, a metabolic end product of Trichomonas vaginalis and Tritrichomonas foetus.** *Mol Biochem Parasitol* 1986, **20**:45-55.

22. Vanacova S RD, Razga J, Hrdy I, Kulda J, et al.: **Iron-induced changes in pyruvate metabolism of *Trichomonas foetus* and involvement of iron in expression of hydrogenosomal proteins.** *Microbiology* 2001, **147**:53-62.

23. Sutak R LE, Tachezy J, Richardson DR (2008): **Crusade for iron: iron uptake in unicellular eukaryotes and its significance for virulence.** *Trends Microbiol* 2008, **16**:261-268.

24. De Jesus JB CP, Junqueira M, Britto C, Silva-Filho FCE, et al. : **A further proteomic study on the effect of iron in the human pathogen *Trichomonas vaginalis*.** *Proteomics* 2007, **7**:1961-1972.

25. De Jesus JB FM, Cuervo P, Britto C, e Silva-Filho FC, et al.: **Iron modulates ecto-phosphohydrolase activities in pathogenic trichomonads.** *Parasitol Int* 2006, **55**:285-290.

26. Beltran NC HL, Jedelsky PL, Sedinova M, Rada P, et al.: **Iron-Induced Changes in the Proteome of *Trichomonas vaginalis* Hydrogenosomes**. *PLoS ONE* 2013, **8**((5)):e65148.

27. Juliano C CP, and Mattana A: **In vitro phagocytic interaction between trichomonas vaginalis isolates and bacteria.** *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology* 1991, **10**((6)):497-502.

28. Rendon-Maldonado JG E-CM, Gonzalez-Robles A, and Martinez-Palomo A.: ***Trichomonas vaginalis*: In vitro phagocytosis of lactobacilli, vaginal epithelial cells, leukocytes, and erythrocytes.** *Experimental Parasitology* 1998, **89**((2)):241-250.

29. Seema S aAK: **An update on trichomonas vaginalis.** *Indian Journal of Sexually Transmitted Diseases and AIDS* 2008, **29**((1)):7-14.

30. Hirt RP NC, Sicheritz-Ponten T, Tachezy J, and Fiori PL.: ***Trichomonas vaginalis* surface proteins: A view from the genome.** *Trends in Parasitology* 2007, **23**((11)):540-547.

31. Bastida-Corcuera FD OC, Colocoussi A, Johnson PJ.: ***Trichomonas vaginalis*** **lipophosphoglycan mutants have reduced adherence and cytotoxicity to human ectocervical cells.** *Eukaryot Cell* 2005, **4**:1951-1958.

32. Singh BN HG, Lucas JJ, Sommer U, Viseux N, Mirgorodskaya E, et al: **Structural details and composition of *Trichomonas vaginalis* lipophosphoglycan in relevance to the epithelial immune function.** . *Glycoconj J* 2009, **26**:3-17.

33. Okumura CY BL, Johnson PJ.: **Galectin-1 on cervical epithelial cells is a receptor for the sexually transmitted human parasite Trichomonas vaginalis.** *Cell Microbiol* 2008, **10**((10)):2078-2090.

34. Hirt RP: ***Trichomonas vaginalis*** **virulence factors : an integrative overview**. *Sex Transm Infect Published* 2013, **89**((6)):439-443.

35. Carlton JM HR, Carlton JM1, Hirt RP, Silva JC, Delcher AL, Schatz M, Zhao Q, Wortman JR, Bidwell SL, Alsmark UC, Besteiro S, Sicheritz-Ponten T, Noel CJ, Dacks JB, Foster PG, Simillion C, Van de Peer Y, Miranda-Saavedra D, Barton GJ, Westrop GD, Müller S, Dessi D, Fiori PL, Ren Q, Paulsen I, Zhang H, Bastida-Corcuera FD, Simoes-Barbosa A, Brown MT, Hayes RD, Mukherjee M, Okumura CY, Schneider R, Smith AJ, Vanacova S, Villalvazo M, Haas BJ, Pertea M, Feldblyum TV, Utterback TR, Shu CL, Osoegawa K, de Jong PJ, Hrdy I, Horvathova L, Zubacova Z, Dolezal P, Malik SB, Logsdon JM Jr, Henze K, Gupta A, Wang CC, Dunne RL, Upcroft JA, Upcroft P, White O, Salzberg SL, Tang P, Chiu CH, Lee YS, Embley TM, Coombs GH, Mottram JC, Tachezy J, Fraser-Liggett CM, Johnson PJ.: **Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis**. *Science* 2007, **315**(5809):207-212.

36. Gomez C ERM, Calixto-Galvez M, Medel O, Rodríguez MA: **Regulation of Gene Expression in Protozoa Parasites**. *Journal of Biomedicine and Biotechnology* 2010, **Volume 2010**(doi:10.1155/2010/726045).

37. Mendoza-Lopez MR B-GC, Fattel-Facenda LV, Avila-Gonzalez L, Ruiz-Tachiquin ME, Ortega-Lopez J, and Arroyo, R.: **CP30, a cysteine proteinase involved in *Trichomonas vaginalis* cytoadherence.** *Infection and Immunity* 2000, **68**((9)):4907-4912.

38. de Miguel N LG, Twu O, Chattopadhyayì A, Wohlschlegel J A, and Johnson PJ: **Proteome analysis of the surface of *Trichomonas vaginalis***

reveals novel proteins and strain-dependent differential expression. *Molecular & Cellular Proteomics: MCP* 2010, **9**((7)):1554-1566.

39. Ikegami A HK, Sharma A, Kuramitsu HK: **Multiple functions of the leucine-rich repeat protein LrrA of Treponema denticola.** *Infect Immun* 2004, **72**((8)):4619-4627.

40. Ma L MQ, Cheng W, Sung Y, Tang P, Hu S, and Yu J.: **Involvement of the GP63 protease in infection of *Trichomonas vaginalis*.** *Parasitology Research* 2011, **109**((1)):71-79.

41. Nakjang S ND, Wipat A, Bolam DN, and Hirt RP: **A novel extracellular metallopeptidase domain sgared by animal host-associated mutualistic and pathogenic microbes**. *Plos ONE* 2012, **7**:e30287.

42. Levine M: **The Zincins: Collagen Fiber Processing and Degradation**. *In: Topics in Dental Biochemistry* 2011, **Berlin Heidelberg: Springer Berlin Heidelberg.**:113-128.

43. Fukasawa KM HT, Ono Y, and Hirose J: **Metal preferences of zinc-binding motif on metalloproteases.** *Journal of Amino Acids* 2011, **574816**.

44. Punta M CP, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer ELL, Eddy SR, Bateman A, Finn RD: **The Pfam protein families database**. *Nucleic Acid Research* 2012, **Database**(40):290-301.

45. Sood S KA: **An update on Trichomonas vaginalis**. *Indian J Sex Transm Dis* 2008, **29**:7-14.

46. Goodman RP, Ghabrial SA, Fichorova RN, Nibert ML: **Trichomonasvirus: A new genus of protozoan viruses in the family Totiviridae**. *Archives of Virology* 2011, **156**(1):171-179.

47. Fichorova RN, Lee Y, Yamamoto HS, Takagi Y, Hayes GR, Goodman RP, Chepa-Lotrea X, Buck OR, Murray R, Kula T *et al*: **Endobiont Viruses Sensed by the Human Host - Beyond Conventional Antiparasitic Therapy**. *PLoS ONE* 2012, **7**(11).

48. Clemente JC UL, Parfrey LW, and Knight R: **The impact of the gut microbiota on human health: An integrative view**. *Cell* 2012, **148**((6)):1258-1270.

49. Brotman RM BL, Conrad M, Gajer P, Ault K, Peralta L, Forney LJ, Carlton JM, Abdo Z, Ravel J.: **Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women.** *Sex Transm Dis* 2012, **39**(10):807-812.

50. Koch A, Bilina A, Teodorowicz L, Stary A: **Mycoplasma hominis and Ureaplasma urealyticum in patients with sexually transmitted diseases**. *Wiener Klinische Wochenschrift* 1997, **109**(14-15):584-589.

51. Martin DH ZM, Lillis RA, Myers L, Nsuami MJ, Ferris MJ.: **Unique vaginal microbiota that includes an unknown Mycoplasma-like organism is associated with Trichomonas vaginalis infection.** *J Infect Dis* 2013, **207**(12):1922-1931.

52. Fettweis JM SM, Huang B, Brooks JP, Glascock AL, Sheth NU; Vaginal Microbiome Consortium, Strauss JF 3rd, Jefferson KK, Buck GA.: **An emerging Mycoplasma associated with trichomoniasis, vaginal infection and disease.** *PLoS One* 2014, **9**(10):e110943.

53. Platt R, Warren JW, Edelin KC: **Infection with Mycoplasma hominis in postpartum fever**. *Lancet* 1980, **2**(8206):1217-1220.

54. Cassell GH, Waites KB, Crouse DT: **Perinatal mycoplasmal infections**. *Clinics in Perinatology* 1991, **18**(2):241-262.

55. Paul VK, Gupta U, Singh M, Nag VL, Takkar D, Bhan MK: **Association of genital mycoplasma colonization with low birth weight**. *International Journal of Gynecology and Obstetrics* 1998, **63**(2):109-114.

56. Dessi D, Rappelli P, Diaz N, Cappuccinelli P, Fiori PL: **Mycoplasma hominis and Trichomonas vaginalis: A unique case of symbiotic relationship between two obligate human parasites**. *Frontiers in Bioscience* 2006, **11**(SUPPL. 1):2028-2034.

57. Varki A aSN: **Historical Background and Overview**. *In: Varki A, Cummings RD, Esko JD,et al, editors Essentials of Glycobiology 2nd edition Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press;* 2009, **Chapter 1.**:Available from: http://www.ncbi.nlm.nih.gov/books/NBK1931.

58. Ouwerkerk JP dVW, and & Belzer C.: **Glycobiome: Bacteria and mucus at the epithelial interface.** *Best Practice & ResearchClinical Gastroenterology* 2013, **27**(1):25-38.

59. Aquino RS LS, Park PW: **Diverse functions of Glycosaminoglycans nin Infection Diseases**. *Progress in Molecular Biology and Translational Science* 2010, **93**:373-393.

60. Tiwari V ME, Sigar IM, Ramsey KH, Shukla D: **Role of heparan sulfate in sexually transmitted infections**. *Glycobiology* 2012, **22**(11):1402-1412.

61. Rabenstein DL: **Heparin and heparan sulphate: structure and function**. *NatProdRep* 2002, **19**:312-331.

62. Sasisekharan R and Venkataraman G: **Heparin and heparan sulphate: biosynthesis, structure and function**. *Curr Opin Chem Biol* 2000, **4**(6):626-631.

63. Kolset SO. TH: **Serglycin--structure and biology**. *Cellular and Molecular Life Sciences : CMLS* 2008, **65**(7-8):1073-1085.

64. Sarrazin S LW, Esko JD.: **Heparan sulfate proteoglycans.** *Cold Spring Harb Perspect Biol* 2011, **3**(7):a004952.

65. Iozzo RV: **Heparan sulfate proteoglycans: intricate molecules with intriguing functions.** *J Clin Invest* 2001, **108**(2):165-167.

66. Mythreye K BG: **Proteoglycan signaling co-receptors: roles in cell adhesion, migration and invasion.** *Cell Signal* 2009, **21**(11):1548-1558.

67. Fitzgerald ML WZ, Park PW, Murphy G, Bernfield M.: **Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase.** *J Cell Biol* 2000, **148**(4):811-824.

68. Endo K TT, Miyamori H, Kinsen H, Yoshizaki T, Furukawa M, Sato H.: **Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration.** *J Biol Chem* 2003, **278**(42):40764-40770.

69. Park PW PG, Preston MJ, Goldberger O, Fitzgerald ML, Bernfield M.: **Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of Pseudomonas aeruginosa.** *J Biol Chem* 2000, **275**(5):3057-3064.

70. Chen Y HA, Bennett AE, Hollingshead SK, Park PW.: ***Streptococcus pneumoniae*** **sheds syndecan-1 ectodomains through ZmpC, a metalloproteinase virulence factor.** *J Biol Chem* 2007, **282**(1):159-167.

71. Ramani VC PP, Thompson CA, DeLucas LD, Sanderson RD.: **Heparan sulfate chains of syndecan-1 regulate ectodomain shedding.** *J Biol Chem* 2012, **287**(13):9952-9961.

72. Tkachenko E RJ, Simons M.: **Syndecans: new kids on the signaling block.** *Circ Res* 2005, **96**(5):488-500.

73. Manon-Jensen T MH, Couchman JR: **Mapping of matrix metalloproteinase cleavage sites on syndecan-1 and syndecan-4 ectodomains.** *FEBS J* 2013, **280**(10):2320-2334.

74. Li Q PP, Wilson CL, Parks WC.: **Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury.** *Cell* 2002, **111**(5):635-646.

75. Bishop JR SM, Esko JD.: **Heparan sulphate proteoglycans fine-tune mammalian physiology.** *Nature* 2007, **446**(7139):1030-1037.

76. Park PW FT, Nishi E, Duncan SJ, Klagsbrun M, Chen Y.: **Activation of syndecan-1 ectodomain shedding by Staphylococcus aureus alpha-toxin and beta-toxin.** *J Biol Chem* 2004, **279**(1):251-258.

77. Nagase H VR, and Murphy G,: **Structure and function of matrix metalloproteinases and TIMPs**. *Cardiovascular Research* 2006, **69**:526-573.

78. Das S MM, Chakraborti T, Mandal A, ans Chakraborti S: **Structure and evolutionary aspects of matrix metalloproteinases: A brief overview**. *Molecular & Cellular Biochemistry* 2003, **253**:31-40.

79. Poincloux R LF, and Chavrier P,: **Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia**. *J Cell Sci* 2009, **122**:3015–3024.

80. Sato H TT, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M.: **A matrix metalloproteinase expressed on the surface of invasive tumour cells.** *Nature* 1994, **370**(6484):61-65.

81. Fillmore HL VT, Broaddus WC: **Membrane-type matrix metalloproteinases (MT-MMPs): expression and function during glioma invasion.** *J Neurooncol* 2001, **53**(2):187-202.

82. Zarrabi K DA, Li J, Kuscu C, Pulkoski-Gross A, Zhi J, Hu Y, Sampson NS, Zucker S, Cao J.: **Inhibition of matrix metalloproteinase 14 (MMP-14)-mediated cancer cell migration.** *J Biol Chem* 2011, **286**(38):33167-33177.

83. Velasco-Loyden G AJ, López-Casillas F.: **The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1.** *J Biol Chem* 2004, **279**(9):7721-7733.

84. Thathiah A CD: **MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17.** *Biochem J* 2004, **382**:363-373.

85. Ficko-Blea E aBA: **The interaction of a carbohydrate-binding module from a clostridium perfringens N-acetyl-beta-hexosaminidase with its carbohydrate receptor.** *The Journal of Biological Chemistry,* 2006, **281**(49):37748-37757.

86. Maestre-Reyna M DR, Veelders MS, Eulenburg G, Kalugin V, Bruckner S, and Essen LO.: **Structural basis for promiscuity and specificity during candida glabrata invasion of host epithelia.** *Proceedings of the National Academy of Sciences of the United States of America,* 2012, **109**(42):16864-16869.

87. Dessì D, Delogu G, Emonte E, Catania MR, Fiori PL, Rappelli P: **Long-term survival and intracellular replication of Mycoplasma hominis in Trichomonas vaginalis cells: Potential role of the protozoon in transmitting bacterial infection**. *Infection and Immunity* 2005, **73**(2):1180-1186.

88. Rappelli P, Carta F, Delogu G, Addis MF, Dessì D, Cappuccinelli P, Fiori PL: **Mycoplasma hominis and Trichomonas vaginalis symbiosis: Multiplicity of infection and transmissibility of M. hominis to human cells**. *Archives of Microbiology* 2001, **175**(1):70-74.

89. Vancini RG, Pereira-Neves A, Borojevic R, Benchimol M: **Trichomonas vaginalis harboring Mycoplasma hominis increases cytopathogenicity in vitro**. *European Journal of Clinical Microbiology and Infectious Diseases* 2008, **27**(4):259-267.

90. Fiori PL, Diaz N, Cocco AR, Rappelli P, Dessi D: **Association of Trichomonas vaginalis with its symbiont Mycoplasma hominis synergistically upregulates the in vitro proinflammatory response of human monocytes**. *Sexually Transmitted Infections* 2013, **89**(6):449-454.

91. Waites K TD: **New developments in human diseases due to mycoplasmas**. *Blanchard A, Browning GF, eds Mycoplasmas molecular biology pathogenicity and strategies for control* 2005, **Norfolk: Horizon bioscience**:289-354.

92. Razin S YD, and Naot Y.: **Molecular Biology and Pathogenicity of Mycoplasmas**. *MICROBIOLOGY AND MOLECULAR BIOLOGY* 1998, **32**:1094-1156.

93. Pereyre S, Sirand-Pugnet P, Beven L, Charron A, Renaudin H, Barré A, Avenaud P, Jacob D, Couloux A, Barbe V *et al*: **Life on arginine for Mycoplasma hominis: Clues from its minimal genome and comparison with other human urogenital mycoplasmas**. *PLoS Genetics* 2009, **5**(10).

94. Yarlett N, Martinez MP, Moharrami MA, Tachezy J: **The contribution of the arginine dihydrolase pathway to energy metabolism by Trichomonas vaginalis**. *Molecular and Biochemical Parasitology* 1996, **78**(1-2):117-125.

95. Morada M SO, Hampl V, Sutak R, Lam B, Rappelli P, Dessì D, Fiori PL, Tachezy J, Yarlett N.: **Hydrogenosome-localization of arginine deiminase in *Trichomonas vaginalis.*** *Mol Biochem Parasitol* 2011, **176**(1):51-54.

96. Huang KY CY, Fang YK, Cheng WH, Cheng CC, Chen YC, Wu TE, Ku FM, Chen SC, Lin R, Tang P.: **Adaptive responses to glucose restriction enhance cell survival, antioxidant capability, and autophagy of the protozoan parasite *Trichomonas vaginalis.*** *Biochim Biophys Acta* 2014, **1840**(1):53-64.

97. Morada M, Manzur M, Lam B, Tan C, Tachezy J, Rappelli P, Dessì D, Fiori PL, Yarlett N: **Arginine metabolism in Trichomonas vaginalis infected with Mycoplasma hominis**. *Microbiology* 2010, **156**(12):3734-3743.

98. Park G, Ryu J, Min D: **The role of nitric oxide as an effector of macrophage-mediated cytotoxicity against Trichomonas vaginalis**. *Korean J Parasitol* 1997, **35**(3):189-195.

99. Han IH, Goo SY, Park SJ, Hwang SJ, Kim YS, Yang MS, Ahn MH, Ryu JS: **Proinflammatory cytokine and nitric oxide production by human macrophages stimulated with Trichomonas vaginalis**. *Korean Journal of Parasitology* 2009, **47**(3):205-212.

100. Wink DA MJ: **Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide.** *Free Radic Biol Med* 1998, **25**(4-5):434-456.

101. Brunet LR: **Nitric oxide in parasitic infections.** *Int Immunopharmacol* 2001, **1**(8):1457-1467.

102. Bogdan C RM, Diefenbach A: **The role of nitric oxide in innate immunity**. *Immunological Reviews* 2000, **173**:17-26.

103. MacMicking J wXQ, and Nathan C: **Nitric oxide and macrophage function**. *Annu Rev Immunol* 1997, **15**:232-250.

104. Blanchard A, Yáñez A, Dybvig K, Watson HL, Griffiths G, Cassell GH: **Evaluation of intraspecies genetic variation within the 16S rRNA gene of Mycoplasma hominis and detection by polymerase chain reaction**. *J Clin Microbiol* 1993, **31**(5):1358–1361.

105. Rigden DJ ML, Galperin MY.: **The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules.** *Trends Biochem Sci* 2004, **29**(7):335-339.

106. Schmidtchen A FI, Björck L.: **Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin.** *Mol Microbiol* 2001, **39**(3):708-713

107. Nam EJ and Park PW: **Shedding of cell membrane-Bound proteoglycans**. *Methods Mol Biol* 2012, **836**(291-305).

108. Kitaya K TY, Hayashi T, Taguchi S, Funabiki M, Nakamura Y, Yasuo T.: **Diverse functions of uterine proteoglycans in human reproduction (review).** *Mol Med Rep* 2012, **5**(6):1375-1381.

109. Chen D AB, Chen L, Vaughan ED, Gerald W, Feng Z, Knudsen BS.: **Syndecan-1 expression in locally invasive and metastatic prostate cancer.** *Urology* 2004, **63**(2):402-407.

110. Alsmark C FP, Sicheritz-Ponten T, Nakjang S, Martin Embley T, Hirt RP.: **Patterns of prokaryotic lateral gene transfers affecting parasitic microbial eukaryotes.** *Genome Biol* 2013, **14**(2):R19.

111. Diaz N DD, Dessole S, Fiori PL, Rappelli P.: **Rapid detection of coinfections by Trichomonas vaginalis, Mycoplasma hominis, and Ureaplasma urealyticum by a new multiplex polymerase chain reaction.** *Diagn Microbiol Infect Dis* 2010, **67**(1):30-36.

112. Taylor-Robinson D, Davies HA, Sarathchandra P, Furr PM: **Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy**. *International Journal of Experimental Pathology* 1991, **72**(6):705-714.

113. Dessi D, Rappelli P, Diaz N, Cappuccinelli P, Fiori PL: **Mycoplasma hominis and Trichomonas vaginalis: A unique case of symbiotic relationship between two obligate human parasites**. *Frontiers in Bioscience* 2006, **11**(SUPPL. 1):2028-2034.

114. Barker J, Brown MRW: **Trojan Horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment**. *Microbiology* 1994, **140**(6):1253-1259.

115. Yarlett N, Lindmark DG, Goldberg B, Moharrami MA, Bacchi CJ: **Subcellular localization of the enzymes of the arginine dihydrolase pathway in Trichomonas vaginalis and Tritrichomonas foetus**. *Journal of Eukaryotic Microbiology* 1994, **41**(6):554-559.

116. Rottem S, Naot Y: **Subversion and exploitation of host cells by mycoplasmas**. *Trends in Microbiology* 1998, **6**(11):436-440.

117. Scholtyseck E, Teras J, Kasakova I, Sethi KK: **Electron microscope observations on the interaction of Mycoplasma fermentans with Trichomonas vaginalis**. *Zeitschrift fur Parasitenkunde* 1985, **71**(4):435-442.

118. Street DA, Wells C, Taylor-Robinson D, Ackers JP: **Interaction between Trichomonas vaginalis and other pathogenic micro-organisms of the human genital tract**. *Br J Vener Dis* 1984, **60**(1):31-38.

119. Bogdan C: **Nitric oxide and the immune response.** *Nat Immunol* 2001, **2**(10):907-916.

120. Sarti P FP, Forte E, Rappelli P, Teixeira M,Mastronicola D, Sanciu G, Giuffre´ A. & Brunori M. : ***Trichomonas vaginalis*** **degrades nitric oxide and expresses a flavorubredoxin-like protein: a new pathogenic mechanism?** . *Cell Mol Life Sci* 2004, **61**:618-623.

Appendices

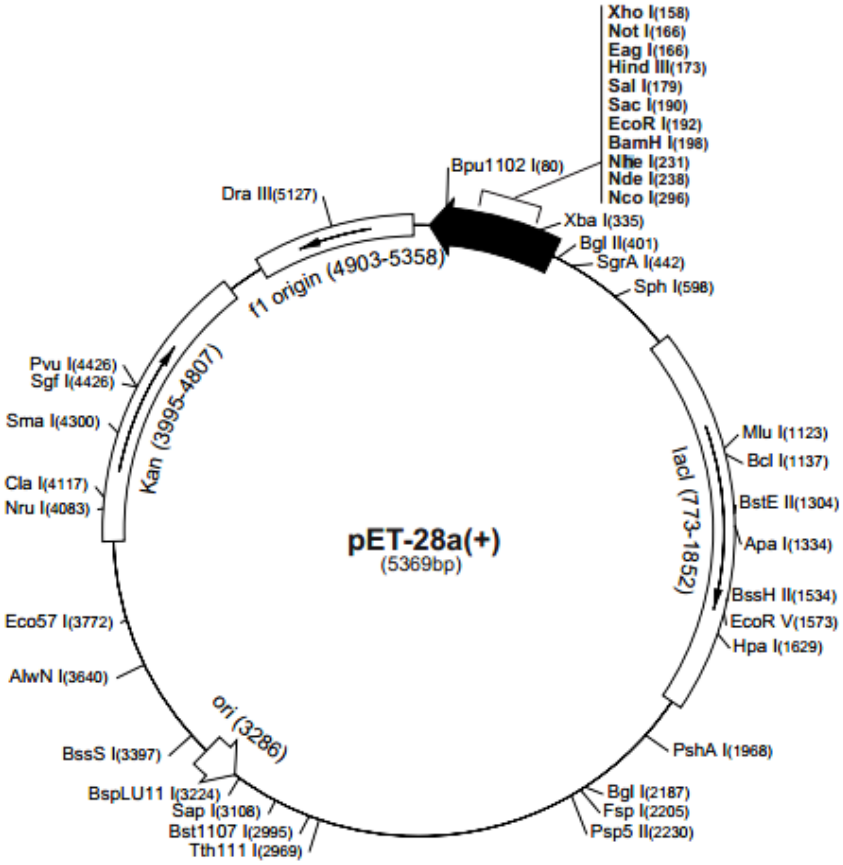
Appendix A: Cloning primers

Recombinant protein code	Forward *BamHI*	Reverse *XhoI*
TvM60L	CGCG GGATCC TATCATGGCATTAATACCGTTCAG	CCGG CTCGAG TTATTTTTCGCCGTTCACTTTGTCTTTC
TvPA14-GBDL	CGCG GGATCC ATGCACGCCTTTGAGTTTGATG	CCGG CTCGAG TTATTTCACGTTAAAGGTCAGTTTACAACC
TvGBDL	CCGC GGATCC GATGTTGATCATATCTTTAAACCG	CCGG CTCGAG TTATTTCACGTTAAAGGTCAGTTTACAACC

Appendix B: Extinction coefficients

Code name for recombinant protein	Extinction coefficient ($M^{-1}cm^{-1}$)
TvM60L	130110
TvPA14-GBDL	52760
TvGBDL	12170

Appendix C: Vector map of pET-28.a


Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Nhe I(231)
Nde I(238)
Nco I(296)
Bpu1102 I(80)
Xba I(335)
Bgl II(401)
SgrA I(442)
Sph I(598)
Dra III(5127)
f1 origin (4903-5358)
Pvu I(4426)
Sgf I(4426)
Sma I(4300)
Kan (3995-4807)
Cla I(4117)
Nru I(4083)
pET-28a(+)
(5369bp)
Mlu I(1123)
Bcl I(1137)
BstE II(1304)
Apa I(1334)
lacI (773-1852)
BssH II(1534)
EcoR V(1573)
Hpa I(1629)
Eco57 I(3772)
AlwN I(3640)
ori (3286)
BssS I(3397)
BspLU11 I(3224)
Sap I(3108)
Bst1107 I(2995)
Tth111 I(2969)
PshA I(1968)
Bgl I(2187)
Fsp I(2205)
Psp5 II(2230)