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**DETECTION and**  
**CHARACTERIZATION of NOVEL**  
**PROTEINS in**  
***TRYPANOSOMA CRUZI***

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Institutet**

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**Cover Image:** A DIC (differential interference contract) microscopy image of a *Trypanosoma cruzi* epimastigote.

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*To my parents, Felisa & Pablo  
To my grandparents, Francisca & Carlos  
With all my deepest for ever love!*



## ABSTRACT

*Trypanosoma cruzi* is a flagellated protozoan parasite. It infects a wide range of mammals, including humans. Human *T. cruzi* infections are endemic to South and Central America. The parasite is transmitted to humans mainly through an insect from the Triatominae subfamily, which feeds from mammals and defecates at the site of the wound, which allows the parasites in the faeces to infect the damaged cells at the bite area. The disease is named Chagas disease in honour of Carlos Chagas who first detected the parasites in the insect, and in this way determined that the vector of the disease was the triatomine bug.

There is no cure for Chagas disease and if it is left untreated it can be lethal in the initial stage of the infection, especially for children. If the affected patients develop the chronic form of the disease, there is a high risk of organ deterioration due to the long term presence of the parasites. Only two drugs are used at present to treat Chagas disease, Nifurtimox and Benznidazole. These drugs were developed in 1960s-70s and they can cause severe side effects. Although preventive and control measures have been effective to reduce transmission, there are still 15,000 deaths per year and over 20 million people are at risk of contracting the disease. Chagas disease is one of the so-called neglected tropical diseases, for which there is little interest from pharmaceutical companies to develop new drugs. In response to the critical need for new safe and effective drugs, much research has been performed by many groups in the field, in order to expand the knowledge on *T. cruzi* biology and to study different enzymes and metabolic pathways that differ from humans.

My aim in this thesis was to detect novel proteins in *T. cruzi* and characterize them by means of: assessing their localization, determining their enzymatic activity, inferring putative identity, if unknown by homology comparisons, and determine if they were really expressed in the parasite.

In paper I, we detected a polyprenyl synthase in the *T. cruzi* EST database. We have expressed and characterized this protein. The enzymes of the polyprenyl synthase family are involved in the synthesis of isoprenoids, which are essential for cell function. The identified protein was a solanesyl diphosphate synthase (TcSPPS) that had all the conserved motifs of the family, presented polyprenyl synthase activity and synthesized the maximum chain isoprenoid, solanesyl diphosphate. Long chain isoprenoids are used in ubiquinone biosynthesis. This was shown by the ability of TcSPPS to complement an *E. coli* strain deficient for ubiquinone production. By using immunofluorescence microscopy, immunogold electron microscopy and cell fractionation we localized TcSPPS to the glycosomes, a peroxisome-like organelle of *T. cruzi*.

In paper II, we report the localization of a short polyprenyl synthase, farnesyl diphosphate synthase (FPPS) in both *T. cruzi* and *Trypanosoma brucei*, a closely related trypanosome to *T. cruzi* that causes sleeping sickness in Africa. Short chain polyprenyl synthases produce short isoprenoids that are utilized to for example modify signalling proteins that utilize the isoprenoid arm to attach to membranes and receptors. As we found the TcSPPS in the glycosomes, we

wanted to determine if the entire part of the isoprenoid pathway where these two enzymes take part, was compartmentalized to the glycosomes or not. We found that this was not the case. Both TcFPPS and TbFPPS are present in the cytoplasm.

In paper III, we partially characterized a third polyprenyl synthase of *T. cruzi*, TcPPS. We detected this protein by western blots analysis in the three stages of the parasite and in the cytoplasm of epimastigotes in *T. cruzi*. It is an unusual protein, due to the 747 amino acid sequence and a molecular weight of 85 kDa, compared to the usual size for the family, which is around 40 kDa. Another particular feature was the presence of a domain of unknown function, DUF2006, which is unrelated to the conserved polyprenyl synthase conserved motifs. We hypothesized that this enzyme could be a GGPPS. The recombinant TcPPS had polyprenyl activity, but the preferred substrate was GPP instead of FPP, the preferred substrates of GGPPSs.

In paper IV, we used a mass spectrometry based proteomic approach to detect novel proteins in an organelle enriched sample from *T. cruzi* epimastigotes. The organellar proteins were separated by 2DGE and 1DGE and subsequently subjected to LC-MS/MS. The results from mass spectrometry were used to search against the *T. cruzi* translated genome. The search rendered 396 protein identifications. For 173 of them, this was the first expression data reported in *T. cruzi*. Furthermore, the proteins in the sample belonged to several organellar compartments and the level of cytoplasmic and highly abundant surface proteins was much reduced.

We located five novel proteins to the acidocalcisome, mitochondrion, ER and cytoplasmic vesicles, through immunofluorescence microscopy of epitope-tagged over-expressed clones.

In summary, this work has contributed to the detection and characterization of several novel proteins in *T. cruzi*, and has answered various questions and has generated new hypotheses to be tested.

## LIST OF PUBLICATIONS

- I. **A solanesyl-diphosphate synthase localizes in glycosomes of *Trypanosoma cruzi*.**

**Marcela Ferella**, Andrea Montalvetti, Peter Rohloff, Kildare Miranda, Jianmin Fang, Silvia Reina, Makoto Kawamukai, Jaqueline Búa, Daniel Nilsson, Carlos Pravia, Alejandro Katzin, Maria Belen Cassera, Lena Aslund, Björn Andersson, Roberto Docampo\*, Esteban J Bontempi\*.

\*Corresponding authors.

J Biol Chem. 2006 Dec 22; 281(51):39339-48.

- II. **Farnesyl Diphosphate Synthase Localizes to the Cytoplasm of *Trypanosoma cruzi* Epimastigotes and *T. brucei* Procyclic Trypomastigotes.**

**Marcela Ferella\***, Zhu-Hong Li, Björn Andersson, Roberto Docampo

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Experimental Parasitology - In Press - Available online 13 March 2008

- III. **An Unusual Polyprenyl Diphosphate Synthase from *Trypanosoma cruzi*.**

**Marcela Ferella**, Tamara Piñero, Patricia Respuela, Ellen Sherwood, Hamid Darban, Bjorn Andersson, Carlos Pravia, Esteban Bontempi\*

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Manuscript - Submitted

- IV. **Proteomics in *Trypanosoma cruzi* - Localization of Novel Proteins to Various Organelles.**

**Marcela Ferella\***, Daniel Nilsson, Hamid Darban, Claudia Rodrigues, Esteban J Bontempi, Roberto Docampo, Björn Andersson

\*Corresponding author.

Proteomics - In Press.

## ADDITIONAL PUBLICATIONS

### **I. Histone acetylation and methylation at sites initiating divergent polycistronic transcription in *Trypanosoma cruzi*.**

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**J Biol Chem - 2008 Apr 9 [Epub ahead of print]**

### **II. Synthesis and biological evaluation of 2-alkylaminoethyl-1,1-bisphosphonic acids against *Trypanosoma cruzi* and *Toxoplasma gondii* targeting farnesyl diphosphate synthase.**

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# TABLE OF CONTENTS

|       |   |        |
|-------|---|--------|
| 1     | Parasites & Parasitism .....  | - 1 -  |
| 1.1   | Parasitic Protozoa .....  | - 1 -  |
| 1.2   | Common Parasitic Protozoan Infections .....                                       | - 1 -  |
| 2     | Trypanosomiases.....  | - 3 -  |
| 2.1   | Chagas Disease .....  | - 3 -  |
| 2.1.1 | Brief History .....   | - 3 -  |
| 2.1.2 | <i>Trypanosoma cruzi</i> Life Cycle .....   | - 3 -  |
| 2.1.3 | Transmission.....   | - 5 -  |
| 2.1.4 | Clinical aspects .....  | - 6 -  |
| 2.1.5 | Diagnosis .....   | - 7 -  |
| 2.1.6 | Burden and Prevention - WHO Initiatives.....                                      | - 7 -  |
| 2.1.7 | Current treatment .....   | - 9 -  |
| 2.2   | African Sleeping Sickness .....   | - 10 - |
| 2.2.1 | <i>Trypanosoma brucei</i> Life Cycle.....   | - 10 - |
| 2.2.2 | Clinical Aspects .....  | - 11 - |
| 2.2.3 | Treatment.....  | - 11 - |
| 3     | Molecular Biology of Trypanosomes.....  | - 12 - |
| 3.1   | Organelles and pathways .....   | - 12 - |
| 3.1.1 | Mitochondrion and Kinetoplast .....   | - 13 - |
| 3.1.2 | Flagellar pocket and Cytostome.....   | - 14 - |
| 3.1.3 | The Reservosome .....   | - 15 - |
| 3.1.4 | GPI-anchored proteins and Antigenic variation....                                 | - 15 - |
| 3.1.5 | Polycistronic transcription and Trans-splicing.....                               | - 16 - |
| 3.1.6 | The Acidocalcisome and Contractile vacuole.....                                   | - 16 - |
| 3.1.7 | Glycosomes – Redox & Energy balance in Carbohydrate metabolism .....              | - 18 - |
| 4     | Chemotherapeutics for Chagas Disease.....   | - 20 - |
| 4.1   | Metabolic Pathways and Proteins under Drug Target evaluation-                     | 20 -   |
| 4.1.1 | Polyamine biosynthesis.....   | - 20 - |
| 4.1.2 | Antioxidant metabolism and Trypanothione reductase -                              | 21 -   |
| 4.1.3 | Proteases - Cystein protease.....   | - 21 - |
| 4.1.4 | Purine synthesis - Hypoxanthine-guanine phosphoribosyltransferase .....           | - 21 - |
| 4.1.5 | GPI-biosynthetic pathway .....  | - 22 - |
| 4.1.6 | Sterol Biosynthetic Pathway – Polyprenyl synthases-                               | 22 -   |
| 5     | Isoprenoid Biosynthesis Pathway .....   | - 24 - |
| 5.1   | Polyprenyl diphosphate Synthases.....   | - 25 - |
| 5.1.1 | Sequence conservation among all- <i>E</i> -polyprenyl diphosphate synthases ..... | - 26 - |
| 6     | Gene-Protein Identifications.....   | - 28 - |
| 7     | <i>Trypanosoma cruzi</i> Genome .....   | - 30 - |
| 7.1   | Phylogeny .....   | - 30 - |
| 7.2   | Genome Project.....   | - 30 - |
| 8     | Proteomics .....  | - 32 - |
| 8.1   | Mass Spectrometry based Proteomics .....  | - 32 - |
| 8.1.1 | The Sample.....   | - 33 - |

|       |  |        |
|-------|--|--------|
| 8.1.2 | Result interpretation and data-handling.....                   | - 34 - |
| 8.2   | Organellar Proteomics .....                                    | - 34 - |
| 9     | Thesis Research.....   | - 36 - |
| 9.1   | Objectives .....   | - 36 - |
| 9.2   | Results and Discussion .....                                   | - 36 - |
| 9.2.1 | Polyprenyl Diphosphate Synthases in <i>Trypanosoma cruzi</i> - | 36 -   |
| 9.2.2 | Large scale proteomics in <i>T. cruzi</i> .....                | - 39 - |
| 9.2.3 | Conclusions .....  | - 40 - |
| 10    | References .....   | - 42 - |

## LIST OF ABBREVIATIONS

|                  |  |
|------------------|--|
| 2DGE             | Two dimensional gel electrophoresis                      |
| 2DLC             | Two dimensional liquid chromatography                    |
| AdoMetDC         | S-adenosylmethionine decarboxylase                       |
| ADP              | Adenosine diphosphate                                    |
| AIDS             | Acquired Immune Deficiency Syndrome                      |
| ATP              | Adenosine triphosphate                                   |
| BAC              | Bacterial artificial chromosome                          |
| Bz               | Benznidazole   |
| C                | Carbon   |
| Ca <sup>+2</sup> | Calcium  |
| cDNA             | Complementary DNA  |
| Cl               | Chlorine   |
| CLD              | Chain length determination                               |
| CVC              | Contractile vacuole complex                              |
| DMAPP            | Dimethylallyl diphosphate or dimethylallyl pyrophosphate |
| DNA              | Deoxyribonucleic acid                                    |
| DNDi             | Drugs for Neglected Diseases initiative                  |
| DPP              | Decaprenyl diphosphate                                   |
| DPSP             | Decaprenyl diphosphate synthase                          |
| ESI              | Electrospray ionization                                  |
| EST              | Expressed sequence tag                                   |
| ExoUase          | U-specific 3'-5'-exonuclease                             |
| FARM             | First aspartate rich motif                               |
| Fe               | Iron   |
| FPP              | Farnesyl diphosphate                                     |
| FPPS             | Farnesyl diphosphate synthase                            |
| FRET             | Fluorescence resonance energy transfer                   |
| GFPP             | Geranyl farnesyl diphosphate                             |
| GFPPS            | Geranyl farnesyl diphosphate synthase                    |
| GGPP             | Geranylgeranyl diphosphate                               |
| GGPPS            | Geranylgeranyl diphosphate synthase                      |
| GlcNAc-PI        | Glucosaminacetylphosphatidylinositol                     |
| GlcN-PI          | Glucosaminylphosphatidylinositol                         |
| GO               | Gene ontology  |
| GPI              | Glycosylphosphatidylinositol                             |
| GPILs            | Glycoinositol phospholipids                              |
| GPP              | Geranyl diphosphate                                      |
| GPPS             | Geranyl diphosphate synthase                             |
| GR               | Glutathione reductase                                    |
| gRNA             | Guide ribonucleic acid                                   |
| H <sup>+</sup>   | Proton   |
| HAT              | Human African trypanosomiasis                            |
| HepPP            | Heptaprenyl diphosphate                                  |

|        |  |
|--------|--|
| HepPPS | Heptaprenyl diphosphate synthase                     |
| HexPP  | Hexaprenyl diphosphate                               |
| HexPPS | Hexaprenyl diphosphate synthase                      |
| HGPRT  | Hypoxanthine-guanine phosphoribosyltransferase       |
| HMGR   | 3-hydroxy-3-methylglutaryl CoA reductase             |
| HTS    | High throughput screening                            |
| IPP    | Isopentenyl diphosphate or Isopentenyl pyrophosphate |
| K      | Potassium  |
| kDNA   | Kinetoplastid deoxyribonucleic acid                  |
| LC     | Liquid chromatography                                |
| m/z    | Mass to charge ratio                                 |
| MALDI  | Matrix assisted laser desorption ionization          |
| MASP   | Mucin associated protein                             |
| Mb     | Megabase   |
| Mg     | Magnesium  |
| mRNA   | Messenger ribonucleic acid                           |
| MS     | Mass spectrometry                                    |
| MS/MS  | Tandem mass spectrometry                             |
| Na     | Sodium   |
| NAD+   | Nicotinamide adenine dinucleotide, oxidized form     |
| NADH   | Nicotinamide adenine dinucleotide, reduced form      |
| Nf     | Nifurtimox   |
| ODC    | Ornithine decarboxylase                              |
| OPP    | Octaprenyl diphosphate                               |
| OPPS   | Octaprenyl diphosphate synthase                      |
| OSC    | Oxidosqualene cyclase                                |
| P      | Phosphorous  |
| PAGE   | Polyacrylamide gel electrophoresis                   |
| PCR    | Polymerase chain reaction                            |
| PFGE   | Pulse field gel electrophoresis                      |
| PFT    | Protein farnesyltransferases                         |
| Pi     | Inorganic phosphate                                  |
| pI     | Isoelectric point                                    |
| Pol II | Polymerase II  |
| Poly-P | Polyphosphate  |
| PPase  | Inorganic pyrophosphatase                            |
| PPDK   | Pyruvate phosphate dikinase                          |
| PPi    | Inorganic pyrophosphate                              |
| PPK    | Polyphosphate kinase                                 |
| PPS    | Polyprenyl diphosphate synthase                      |
| PPX    | Exopolyphosphatase                                   |
| Q      | Quinone  |
| RNA    | Ribonucleic acid                                     |
| RNAi   | Interference RNA                                     |
| S      | Sulfur   |
| SAGE   | Serial analysis of gene expression                   |
| SARM   | Second aspartate rich motif                          |
| SBI    | Sterol Biosynthesis Inhibitors                       |

|                  |  |
|------------------|--|
| SL               | Splice Leader  |
| SPP              | Solanesyl diphosphate  |
| SPPS             | Solanesyl diphosphate synthase                                   |
| SQS              | Squalene synthase  |
| <i>T. b.</i>     | <i>Trypanosoma brucei</i>  |
| <i>T. brucei</i> | <i>Trypanosoma brucei</i>  |
| <i>T. cruzi</i>  | <i>Trypanosoma cruzi</i>   |
| TbVSP1           | Soluble vacuolar pyrophosphatase from <i>T. brucei</i>           |
| Tc               | <i>Trypanosoma cruzi</i>   |
| TDR              | Special Programme for Research and Training in Tropical Diseases |
| TR               | Trypanothione reductase  |
| TS               | Trypanothione synthetase   |
| TS               | Trans-sialidase  |
| TUTase           | 3' Terminal uridyl transferase                                   |
| U                | Uridine  |
| UPP              | Undecaprenyl diphosphate   |
| UPPS             | Undecaprenyl diphosphate synthase                                |
| UQ               | Ubiquinone   |
| VSG              | Variant surface glycoprotein                                     |
| VTC              | Vacuolar transporter chaperone                                   |
| WGS              | Whole genome shotgun   |
| WHO              | World Health Organization  |
| YAC              | Yeast artificial chromosome                                      |
| Zn               | Zinc   |

# 1 PARASITES & PARASITISM

A **parasite** is an organism that cannot live independently. It lives in or on another organism (the host) and makes use of the environment and resources of the host.

**Parasitism** is a type of symbiotic relationship between organisms of different species. One organism, the parasite, benefits from a prolonged, close association with the other organism, the host, which is harmed.

The parasites switch between different environments through their life cycles. Inside the host, the parasite needs to adapt in order to survive to the defence mechanisms of the immune response (Markell 1999). Interference with the vital processes of the host through the action of secretions, excretions or other products of the parasite, are the parasite means to succeed. Parasites affect different aspects of the host immune system or defenses, its metabolism, its resources and they may even modify their behavior.

## 1.1 PARASITIC PROTOZOA

Protozoa are a variable group of single celled eukaryotes. **Parasitic protozoa** are parasites that affect plants, invertebrates and most vertebrates including humans. They are widely spread in nature to the extent that every organism at some point in its life is inhabited by these single celled parasites.

But the protozoan parasites are not as simple as they might seem. Their life cycles and adaptations are frequently very complex and specialized. In humans, colonization by some of these parasites ends in a parasitic infection or disease.

## 1.2 COMMON PARASITIC PROTOZOAN INFECTIONS

- Amoebiasis

Amoebae: Unicellular non-flagellated organisms with pseudopodia used for feeding and locomotion; obligate parasites (Cox 2005).

Several species of intestinal amoebae colonize humans worldwide but only one is pathogenic, *Entamoeba histolyca*. It invades the small intestine and colon and can spread to the liver and other parts in the body causing amoebic dysentery or hepatic amoebiasis (liver abscesses).

- Intestinal and Urogenital Infections

Intestinal Flagellates: This group includes single celled organisms that lack plastids, mitochondria, Golgi and other organelles, such as peroxisomes (Cox 2005).

*Giardia lamblia*, a binucleated flagellated protozoan parasite, causes diarrhea throughout the world. It is still not known how many species infect humans. *Giardia* represents one of the earliest diverging lineages among eukaryotes.

Trichomonads are parasites that affect the urogenital tract and cause sexually transmitted diseases in humans (*Trichomonas vaginalis*) and animals. Its

characteristic organelle is the hydrogenosome, which was found to be related to the mitochondria.

- Malaria

*Plasmodium spp* are the malaria parasites that infect red blood cells in mammals. They are transmitted through a genus (*Anopheles*) of mosquitoes. Human malaria is one of the most common infectious diseases in tropical and subtropical regions, specially the Sub-Saharan Africa.

- Toxoplasmosis

*Toxoplasma gondii* is an intracellular parasite of humans and animals and a major opportunistic pathogen in AIDS patients. It rarely causes disease but it is dangerous in pregnant women; the parasite can cross the placenta and damage the developing fetus.

- Cryptosporidiosis

Cryptosporidiosis is an enteric disease of medical and veterinary importance caused by coccidian parasites such as *Cryptosporidium parvum*. *Cryptosporidium* are common parasites in the intestinal and respiratory tract of mammals, birds and reptiles. These infections are not pleasant but they are only dangerous in immunocompromised individuals, to whom it can be fatal.

- Trypanosomiasis and Leishmaniasis

Trypanosomiasis and Leishmaniasis are caused by kinetoplastid flagellated parasites.

Leishmaniasis is caused by intracellular parasitic protozoa of the genus *Leishmania*. Humans become infected via the bite of sandflies carrying *Leishmania spp*. The disease can be cutaneous (skin ulcers on exposed areas or ulcers in mucous membranes of the nose, mouth and throat) or visceral known as kala azar (high fever, swelling of spleen and liver and anemia). If visceral leishmaniasis is untreated the fatality rate is maximum within two years (WHO/TDR 2008).

African trypanosomes (*Trypanosoma brucei*) live in the bloodstream and cause sleeping sickness in humans. American trypanosomes (*Trypanosoma cruzi*) alternate between intra and extracellular forms in the host causing Chagas disease.



## 2 TRYPANOSOMIASES

### 2.1 CHAGAS DISEASE

#### 2.1.1 Brief History

Carlos Justiniano Ribeiro Chagas was born in July 1879 in a small town in Minas Gerais, Brazil. He started his medical carrier in 1896 at the Faculty of Medicine in Rio de Janeiro. During those years, Brazil was suffering from many infectious diseases such as yellow fever, smallpox, bubonic plague and malaria. In 1902 Carlos Chagas met Oswaldo Cruz, who was starting a campaign to eradicate yellow fever from the Brazilian ports, which were the most affected areas in the 1900s. Years later he directed prophylactic campaigns against malaria in labor camps, to great success. In 1907 he was designated to direct another campaign in Minas Gerais, where the railway workers were severely affected by malaria (Lewinsohn 1981; Lewinsohn 2003). They were the first ones to mention to Chagas the presence of the “barbeiros” or “blood sucking bugs”.



*“Once we heard of the blood-sucking habits of this insect and of its proliferation in human dwelling-places, we became very interested in knowing its exact biology and above all in ascertaining if by any chance it were, as I immediately supposed, a transmitter of any parasite of man or another vertebrate.” (Chagas 1922)*

It was Carlos Chagas who first discovered flagellates in the midgut of the insects when dissecting the “barbeiros”, and identified them as a new species of trypanosome, different from the already known *Trypanosoma brucei*, the cause of sleeping sickness in Africa. He named the parasite *Trypanosoma cruzi* in honor of his mentor Oswaldo Cruz. Searching for the hosts he came to meet a two year old girl, Berenice, in Minas Gerais, presenting a swollen face and a persistent high fever. When he tested her blood, he found the same flagellated parasites as in the insects he dissect and animals he had tested previously (Lewinsohn 1981; Lewinsohn 2003; Miles 2005). It was the first reported case of acute *T.cruzi* infection in humans (Chagas 1909).

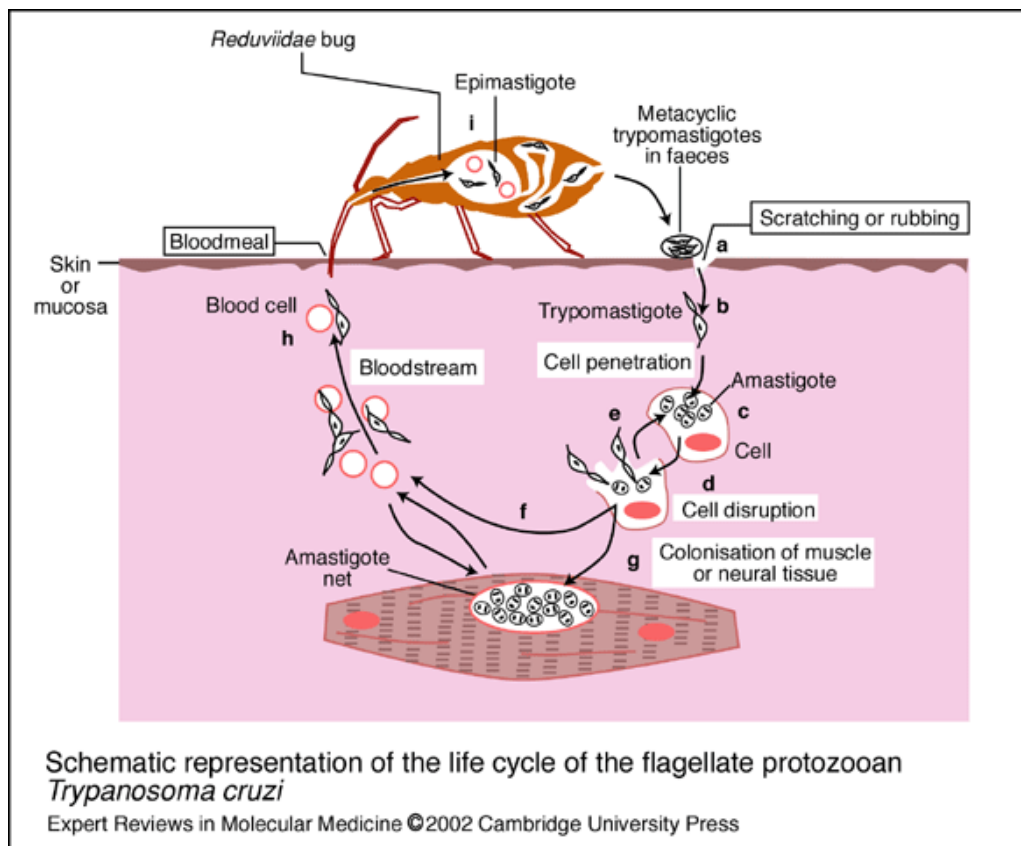
#### 2.1.2 *Trypanosoma cruzi* Life Cycle

The life cycle of *Trypanosoma cruzi* starts when the invertebrate host (the insect that carries the parasites, usually called the vector) feeds from the mammalian host and defecates near the wound. The metacyclic trypomastigotes present in the insect vector feces, invade the cells at site of the insect bite (Figure I).

Invasion of the cells occurs after the parasite attaches to the cell plasma membrane via sialic acid residue recognition, removal and transfer, to proteins

in the parasite plasma membrane (De Souza 2002; Miles 2005). There is a recruitment of lysosomes to the invasion site (Tardieux, Webster et al. 1992; Burleigh 2005), with the consequent formation of a parasitophorous vacuole which allows the entry of the parasite through an endocytic process. The vacuole fuses with the lysosomes to form a phagolysosome, from where the parasite escapes into the cytosol. Here the trypomastigotes transform into amastigotes. This form is able to multiply by binary fission forming a pseudocyst, and around five days later, there could be up to 500 amastigotes inside one pseudocyst, transforming to trypomastigotes; small motile c-shaped cells (Miles 2005). These parasites are released to the intracellular space to infect other cells or continue to the bloodstream where they will circulate. The cycle continues if another bug feeds from the infected individual. Inside the invertebrate host, *T. cruzi* is confined to the alimentary tract where the trypomastigotes ingested transform into amastigotes and sphaeromastigotes and into epimastigotes in the midgut which multiply by binary fission. Once in the rectum, the parasites attach to the epithelium and start to differentiate into metacyclic trypomastigotes ready to be released with the next feeding event (de Souza 1984; Vickerman 1985; Tyler and Engman 2001; De Souza 2002; Miles 2005).

**Figure I: *Trypanosoma cruzi* Life Cycle**



Extracted from (<http://www-ermm.cbcu.cam.ac.uk>) with author's permission.

**Life cycle:** **a.** During feeding, the insect defecates on the skin; **b.** The protozoa in the feces, in the form of metacyclic trypomastigotes enter the mammalian host; **c.** The trypomastigotes penetrate local cells and transform into amastigotes and multiply; **d.** The amastigotes transform

again into trypomastigotes that disrupt the cells and parasites are released; **e.** Circulating parasites infect new cells; **f.** Re-enter the blood stream or **g.** Colonize muscle and neural tissues as amastigote nets; **h.** *Reduviidae* bugs are infected when feeding on contaminated blood; **i.** In the insect's midgut, trypomastigotes change into epimastigotes; replicative forms. Reaching the rectum, these forms differentiate into the infective metacyclic trypomastigotes, thus completing the life cycle (Macedo, Oliveira et al. 2002).

### 2.1.3 Transmission

#### 2.1.3.1 The Vector

Transmission of Chagas disease occurs mainly by the insect vectors carrying the parasite in their digestive tract as described by Chagas in 1909. These insects or barbeiros or sucking-bugs, belong to the family Reduviidae, sub-family Triatominae. There are over 140 species described to date and more continue to be discovered (Miles 2004). All species are potentially able to transmit the disease, but some are poor vectors due to their feeding and defecation behavior. Some species of triatomine defecate longer after they feed, which makes them poor vectors of the parasite. Several other species defecate while feeding and in contact with the host, and these are the main vectors of transmission (Zeledon, Alvarado et al. 1977; Dujardin 2004; Coura 2007). *Triatomine infestans*, is the main vector in southern South America (Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay and south of Peru), while *Rhodnius prolixus* and *Triatoma dimidiata* colonize northern South America and Central America (Miles 2004).

The natural habitats of these bugs were palm trees, burrows, hollow trees, tree holes, beneath tree bark and the nests of some bird species, and the disease was a zoonosis affecting wild animals and rodents. When men invaded these natural environments displacing wild animals and constructing houses, the triatomines had to adapt to the new environment. Several species moved to populated areas and started to feed on domestic animals and humans (Coura 2007). This adaptation of triatomines to human homes is certainly the most important determinant for the establishment of human infection. In this regard, the reduviidae bugs can be divided in three groups: (i) domestic vectors inhabiting houses and rural areas in close proximity to populations, and thus transmitting the parasite to humans; (ii) sylvatic species in the process of adaptation to colonize human environments and are so called candidate vectors; (iii) sylvatic species that remain sylvatic, in contact with wild mammals (reservoirs of trypanosomes) and are potential future vectors (Dujardin 2004).

### 2.1.3.2 Other Routes for Transmission

| Transmission                            |  |
|---|--|
| <b>Blood transfusions</b>               | This is still the second cause of transmission, due to blood donors not being tested. The danger may come not only from whole blood, but from packed red cells, platelets, white cells, fresh frozen plasma, and cryoprecipitate (Schmunis 1999).  |
| <b>Oral transmission</b>                | Ingestion of food contaminated either with entire triatomines or their feces; ingestion of raw meat from infected wild mammals carrying pseudocysts and blood-form trypomastigotes, (Coura, Junqueira et al. 2002); ingestion of drinks such as palm juice (da Silva Valente, de Costa Valente et al. 1999). |
| <b>Congenital transmission</b>          | Mothers can pass the parasites to their baby during pregnancy, at delivery, or while breastfeeding (Punukollu, Gowda et al. 2007).   |
| <b>Organ transplantation</b>            | Transplants became another source of transmission, when infected individuals, unaware of their condition became organ donors. In the recipients, immune-suppressed patients, the disease reactivates (Barcan, Luna et al. 2005).   |
| <b>Accidental laboratory infections</b> | Although rare, this is another source of infection.  |

### 2.1.4 Clinical aspects

There are two phases of Chagas disease: acute and chronic. Both are characterized by specific conditions and levels of circulating parasites.

#### 2.1.4.1 Acute Chagas disease

Soon after the infection takes place, the **acute phase** starts and it can be lethal. It is not usually detected as its symptoms are normally confused with other conditions. Over 90% of the cases are asymptomatic (Teixeira, Nitz et al. 2006). In some cases, there could be a visible lesion at the entry site; a cutaneous chagoma (Miles 2005). If the parasites cross the eye membrane or conjunctiva, the local eye become inflamed; this is known as Romana's sign (Dias 1997).

Fever, myalgias, somnolence, cramps and diarrhea, respiratory disturbances, muscle and joint pains, sweating, hepatosplenomegalia, heart problems such as abnormalities in the electrocardiograph and myocardiopathy (Teixeira, Nitz et al. 2006; Punukollu, Gowda et al. 2007), develop after 7-10 days post infection and disappear after two to three months. Death during acute phase is related to compromised heart, intestines, and nervous system (Teixeira, Nascimento et al. 2006). Children are most affected during this stage, especially when under age of 10.

#### **2.1.4.2 Chronic Chagas disease**

This phase is in most cases asymptomatic, with normal heart, esophagus, and colon. For one third of the chronic individuals (Teixeira, Nitz et al. 2006), after 20-50 years of infection, the disease may evolve to the cardiac, digestive form (cardiac arrest or megaesophagus and megacolon, respectively), or cardiac and digestive forms together (Coura 2007) with “silent” advanced organ and tissue damage. The cardiac form of Chagas disease is the most severe and the most common. Its signs are arrhythmia, cardiac insufficiency, auricular-ventricular and branch blockages and tromboembolism (Coura 2007).

#### **2.1.5 Diagnosis**

The diagnosis of Chagas disease is very much dependent on the parasites circulating in the blood of the infected person. This makes it harder to diagnose infected persons when they are asymptomatic or the levels of parasites in blood are very low, contributing to spread the disease unknowingly. There are several methods of diagnosis used over the years. Direct parasitological methods (Freilij and Altcheh 1995; Luquetti 2004) are used during the acute phase when the levels of parasites in the blood are easily detected. During the chronic phase, parasites in the blood are below the microscope detection threshold and high levels of antibodies against *T. cruzi* antigens circulate in the bloodstream. Diagnosis is based on serological (Levin, Franco da Silveira et al. 1991; Umezawa and Silveira 1999) or indirect methods (Gomes 1997; Luz 1999; Schenone 1999), with the polymerase chain reaction (PCR) as the latest in use for detecting *T. cruzi* (Avila, Pereira et al. 1993; Wincker, Britto et al. 1994).

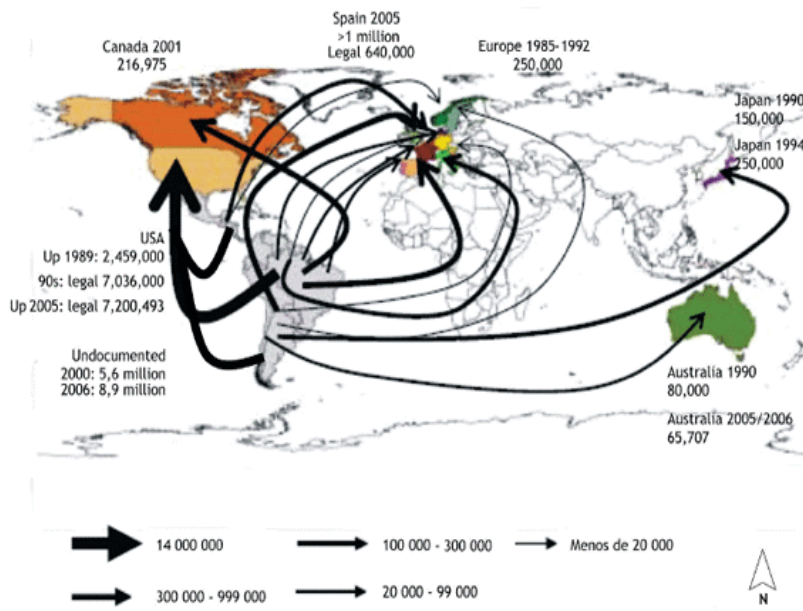
No ideal test is as yet available with high sensitivity, specificity and that is easy to use and inexpensive (Schmunis 2007).

#### **2.1.6 Burden and Prevention - WHO Initiatives**

The population at risk of contracting Chagas disease is estimated to 25 million or more in Central and South America, and there is a continuous risk for 6-8 million of these persons to develop clinical manifestations of the chronic form of Chagas (WHO 2008, [www.who.int](http://www.who.int)), leading to death specially in children and immunocompromised persons. There are still 10,000 to 20,000 deaths per year in endemic areas, and Chagas disease is still considered the second highest burden among Tropical Diseases in the American continent (WHO/TDR 2007).

Cases of blood transmission in the USA and Canada (Young, Losikoff et al. 2007) have been reported, showing that due to increased migration the disease is crossing into developed countries (Schmunis 2007) (Figure II).

**Figure II: Immigration map showing the flow from Latin American countries to non-endemic areas for Chagas disease.** Research project on international migration in Latin America.



Reproduced with permissions from (Schmunis 2007).

The World Health Organization (WHO) has performed initiatives in endemic countries in Latin America as a way to promote efforts in interrupting the transmission of Chagas disease. The focus has been to control the vector through fumigation campaigns, systematic screening of blood donors in all endemic countries, detection and treatment of congenital transmission and treatment of children and acute cases. This effort has involved the regional and national health government authorities. The campaigns inform the population of the real risk and involve the communities for surveillance of the vector and transmission. Although great advances have been made thanks to these measures, to the extent that some countries have declared that they are free of *Triatomine infestans* infections, the main vector in urban areas (WHO/TDR 2007), changes in the socioeconomics of these countries, in several opportunities ended these campaigns.

**Table I:** Decrease in Chagas disease incidence due to interruption of transmission (WHO/TDR 2007)

| Epidemiological parameters | 1990        | 2000       | 2006       |
|----------------------------|-------------|------------|------------|
| Annual deaths              | >45 000     | 21 000     | 12 500     |
| Cases of Human Infection   | 30 million  | 18 million | 15 million |
| New cases per year         | 700 000     | 200 000    | 41 200     |
| Population at risk         | 100 million | 40 million | 28 million |
| Number of countries        | 21          | 21         | 21         |

© Source: TDR/PAHO/WHO. Reproduced with permission.

It is clear the burden is still high and drugs are critically needed to treat both acute and chronic disease, as are safer and more effective drugs adapted to patient needs (ie. paediatric formulation).

### 2.1.7 Current treatment

There is no cure for Chagas disease. After its discovery, several compounds have been tested on patients. Since the end of 1960s and beginning of the 70s, only two drugs have been used for the treatment of Chagas: nifurtimox (Nf) (used for acute & early indeterminate stages of the disease) and benznidazole (Bz) (which requires 60 days of treatment in acute infections and is only effective in 50% of cases) (Coura 2002).

Nifurtimox generates reactive compounds, toxic for the parasite, which is deficient in free radical detoxification pathways (Docampo and Moreno 1984) and Benznidazole interferes with the synthesis of macromolecules by interacting with nitroreduction intermediates (de Castro 1993). When administered in recent infections, the drugs diminish the parasitemia in blood to undetected levels, but the results much depend on the length of the treatment. Severe side effects occur during this period. Nf causes anorexia, psychological alterations, nausea, vomiting, intestinal colic and diarrhea, while Bz has a larger set of adverse reactions. It can cause symptoms of hypersensitivity, dermatitis, fever, articular and muscular pain, depression of bone marrow, agranulocytosis and polyneuritis of peripheral nerves, among others (Coura 2002). Benznidazole is the only drug that is commercially available in several countries.

There are no treatments for indeterminate and chronic stages of the disease (DNDi, [www.dndi.org](http://www.dndi.org)). **DNDi, the Drugs for Neglected Diseases initiative** was formed in 2003 from seven organizations around the world: five public sector institutions – the Oswaldo Cruz Foundation from Brazil, the Indian Council for Medical Research, the Kenya Medical Research Institute, the Ministry of Health of Malaysia and France's Pasteur Institute; one humanitarian organisation, Médecins sans Frontières (MSF); and one international research organisation, the UNDP/World Bank/WHO's Special Programme for Research and Training in Tropical Diseases (TDR), which acts as a permanent observer to the initiative.

This is one of several similar initiatives and joint organizations around the world formed to combat tropical infections and parasitic diseases, normally neglected diseases.

DNDi's main objective is to develop new drugs or new formulations of already existing ones, for the so called neglected diseases, such as sleeping sickness, kala-azar, and Chagas disease ([www.dndi.org](http://www.dndi.org)).

Several metabolic pathways and biological molecules from *T. cruzi* are now under drug target evaluation, by DNDi, by many public-private partnerships and by individual research groups around the world with the common objective of finding a better drug for the treatment and cure of Chagas disease (see Section 4).

## 2.2 AFRICAN SLEEPING SICKNESS

Evidence of African sleeping sickness or Human African trypanosomiasis (HAT) can be traced back to ancient times, up to 2000 BC (Steverding 2008). The first connection was made between the disease and the tsetse fly during the 1800's. But it was not until 1895 that *Trypanosoma brucei* was found to be the cause of cattle sleeping sickness, and the first report of its infection in humans was not until 1901-1902 (Steverding 2008).

HAT or sleeping sickness is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The disease is endemic to sub-Saharan Africa where the tsetse flies (the main vector of transmission) have their habitat constrained by the temperature and humidity of the region (Black 2005).

Some other species of trypanosomes (i. e. *T. congolense*, *T. vivax* and *T. evansi*), infect cattle and other domesticated animals in Africa, causing the disease known as 'nagana', which costs three billion dollars a year in productivity losses. Furthermore, the inability to keep cattle in these areas of Central Africa, inhibits the regional development (Connor 1994).

In 2003, countries reported about 17 000 new cases to WHO. More than 80% of these cases were reported from Angola (3 000 cases) and the Democratic Republic of Congo (11 000 cases) (Pepin and Meda 2001).

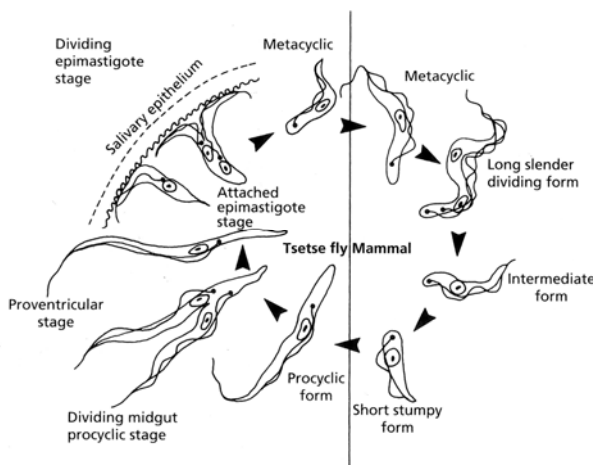
Although the human infections were contained in the 1950's, the incidence now reaches up to 300-500 thousand people with sleeping sickness, which even exceeds the numbers of AIDS patients in some areas (Black 2005).

The World Health Organization (WHO) has launched a series of programs to stop the transmission. Although these measures help contain the transmission, they are always vulnerable to socio-political problems.

### 2.2.1 *Trypanosoma brucei* Life Cycle

*Trypanosoma brucei* has a complex life cycle in the vector (Vickerman, Tetley et al. 1988; Black 2005) (Figure III). The parasites in the midgut migrate to the salivary glands and differentiate into non-infective epimastigotes. This form divides continuously, transforming into infective metacyclic forms. When the tsetse fly bites the mammalian host, the parasites are injected and

transform into bloodstream form trypanosomes. There is an active multiplication process, which generates high levels of parasites, ready to be taken up by another fly and continue the cycle.



**Figure III: *T. brucei* life cycle.**

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## 2.2.2 Clinical Aspects

Although there are differences in the clinical manifestations and epidemiology of sleeping sickness caused by *T.b. rhodesiense* or *T. b. gambiense*, both infections are fatal to humans. *T. b. gambiense* generates a chronic disease that last up to four years, while infections by *T.b. rhodesiense* cause an acute state lasting no longer than nine months before death.

African sleeping sickness can be divided into three phases (Black 2005).

*Phase 1:* Begins with the bite of the tsetse fly. At the site of the bite a chancre occurs where the parasites remain.

*Phase 2:* The trypanosomes spread to the whole body through the blood and lymphatic vessels multiplying in number. During this period, the parasitemia goes up and down, due to the evasion or action of the immune system response, respectively (Hajduk 1984; Dubois, Demick et al. 2005).

*Phase 3:* Infected individuals develop central nervous system disorders, if untreated. This is due to the parasites being able to cross the blood brain barrier (Enanga, Burchmore et al. 2002). The final stage resulting in heart, nervous system and organs damage, involves a complete state of somnolence (sleeping sickness) and eventually death.

## 2.2.3 Treatment

There is neither efficient treatment nor vaccines for Sleeping sickness. Only four drugs are available against *T. brucei* species: Suramin (1920), Pentamidine (1939), Melarsoprol (1949) and the latest, Eflornithine (1991) only effective against *T. b. gambiense*. All four present toxic effects and Melarsoprol started to be ineffective when resistant strains emerged (Nwaka and Hudson 2006; WHO/TDR 2008).

The mode of action of Pentamidine and Suramin is not well known, but both are charged molecules and due to this they cannot cross the brain blood barrier where trypanosomes hide during the late-stage of the disease (Naula and Burchmore 2003). Eflornithine (produced by Sanofi Aventis) appeared to be an alternative for strains resistant to Melarsoprol, useful for the late-stage of sleeping sickness, but this drug has a high production cost and only through WHO funds or donations (Robays, Raguenaud et al. 2008) is administered to endemic areas. Eflornithine is an ornithine analog that inhibits the trypanosomal ornithine decarboxylase (ODC), and thus blocking the polyamine synthesis (McCann and Pegg 1992; Delespaux and de Koning 2007).

In the same manner as for Chagas disease, there is now a main focus on the biology of trypanosomes in order to evaluate several processes, and the enzymes involved, as drug target candidates (Naula and Burchmore 2003). Some processes under study are glycolysis, protein prenylation, sterol metabolism, polyamine metabolism, proteases, membrane transport, among other target pathways.

### 3 MOLECULAR BIOLOGY of TRYPANOSOMES

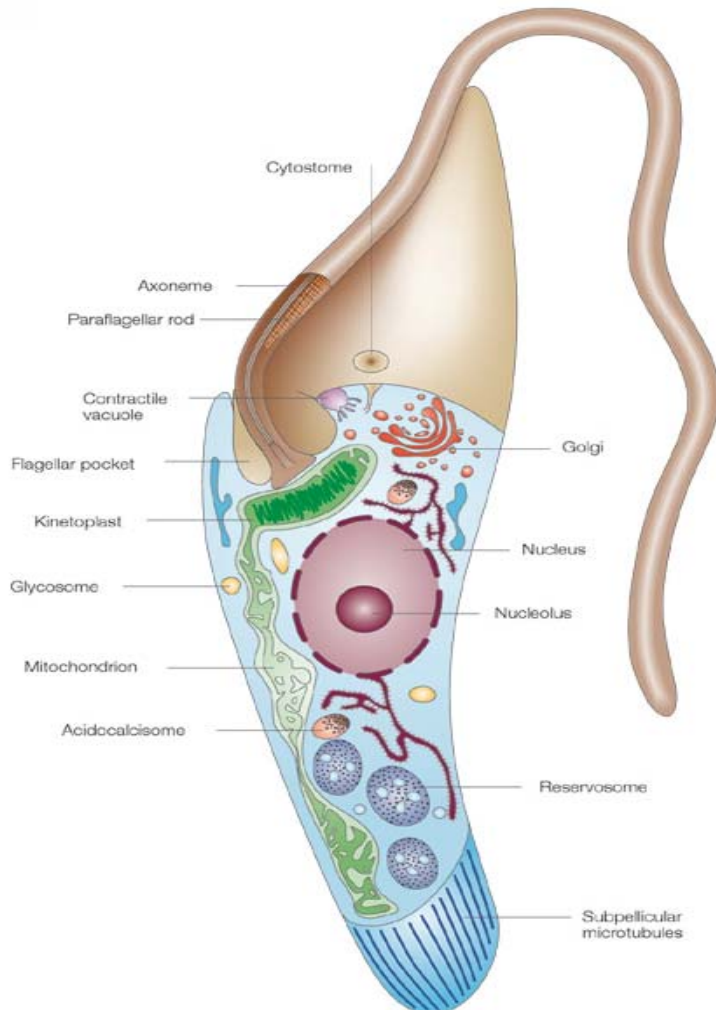
As discussed in previous sections, trypanosomes are important organisms of study due to the diseases they cause. Improved understanding of their biology and specially their discrepancies with the human host, contributes to the rational design of drugs against trypanosomiasis.

However, these parasites are also interesting as model organisms. Trypanosomes possess particular organelles and many unique molecular mechanisms.

#### 3.1 ORGANELLES AND PATHWAYS

Like any other eukaryotic cell, protozoan parasites have particular cytoplasmic structures and organelles. Trypanosomes possess a nucleus, mitochondrion, Golgi, endoplasmic reticulum, peroxisomes and lysosomes which resemble those of other eukaryotes. However these parasites also contain specific organelles such as a kinetoplast, glycosomes (a specialized peroxisome-like organelle), reservosomes, acidocalcisomes, a contractile vacuole, a cytostome and a flagellar pocket. Their processes and metabolic

pathways are also often different from those found in other protozoans and parasites as well as higher eukaryotes.

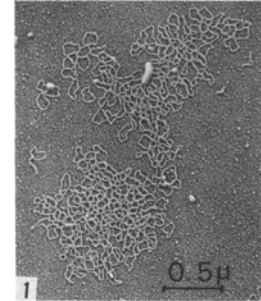


**Figure IV: Scheme of an epimastigote from *Trypanosoma cruzi*** (Docampo, de Souza et al. 2005), (Reprinted with Permission; © Nature Rev. Microbiology).

### 3.1.1 Mitochondrion and Kinetoplast

The **mitochondrion** spreads along the whole cell body of trypanosomes, as a single large organelle that contains the **kinetoplast** (Vickerman and Coombs 1999).

The kinetoplast, although a self duplicating-body and sometimes considered as an independent organelle, is a specialized compact region contained in the mitochondrion. It holds a dense network of DNA in the form of maxi and mini circles (Figure V) (Riou and Delain 1969; Riou and Delain 1969; Shapiro 1993; Chen, Rauch et al. 1995; Shapiro and Englund 1995).



**Figure V: Associations of relaxed circles.** Electron micrographs of kDNA after ethidium bromide treatment (Riou and Delain 1969). ©PNAS (Reprinted with permission).

The maxi circles carry the mitochondrial genes for the oxidative phosphorylation, including the subunits of the respiratory complexes (Stuart, Schnauffer et al. 2005), while the mini circles hold the net of maxi circles together and encode for the guide RNAs (explained below in Section 3.1.1.1).

The mitochondrion, being an energy metabolism organelle, contributes to the adaptation to the changes in environment through the trypanosome life cycle (Hannaert, Bringaud et al. 2003).

#### 3.1.1.1 RNA Editing

The modification of mitochondrial mRNA in trypanosomatids by insertion/deletion of uridine (U) residues (Benne, Van den Burg et al. 1986) is termed RNA editing. It consists of an enzymatic cleavage of the pre-mRNA at the site where it interacts with a guide RNA (gRNA). Consequently, a series of uridines are added or removed by a 3' terminal uridyl transferase (TUTase) or a U-specific 3'-5'-exonuclease (ExoUase), following the gRNAs sequences. Finally the cleaved pre-mRNAs are ligated to render the final message, the processed mRNA (Blum, Bakalara et al. 1990; Blum and Simpson 1990; Simpson, Sbicego et al. 2003; Stuart, Schnauffer et al. 2005). The complex machinery of the editing process, termed "editosome", involves several specific enzymes like the TUTase and ExoUase and proteins of unknown specific function in the process (Simpson, Sbicego et al. 2003).

The editing is also a mutational tool that allows for the switch off and on of mitochondrial genes, which leads to changes in respiration (Vickerman and Coombs 1999).

Although this type of RNA editing mechanism is characteristic of the kinetoplastid protozoans, various types of RNA modifications exist, and have been reported later on in other species, from viruses to plants and humans (Gray and Covello 1993; Lafontaine and Tollervey 1998; Gott and Emeson 2000; Casey 2006).

### 3.1.2 Flagellar pocket and Cytostome

The **flagellar pocket** is the single area of the cell where endocytic and exocytic activities take place (Overath, Stierhof et al. 1997). It is the way for the trypanosomatids to communicate with the environment without exposing themselves. The pocket acts as a restricted area to internalize macromolecules, while avoiding the exposure of the endocytic receptors to the immune system (Webster and Russell 1993; Overath, Stierhof et al. 1997). *Trypanosoma brucei*, delivers to the surface and recycles its variant surface glycoproteins (VSGs) through the pocket when evading or being overcome by the humoral immune response (Seyfang, Mecke et al. 1990; Webster and Russell 1993; Engstler, Thilo et al. 2004).

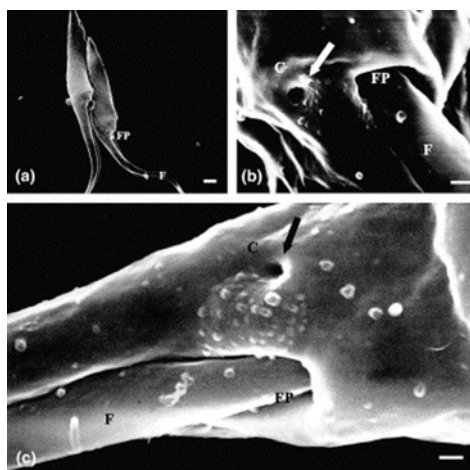
The flagellar pocket is formed by an invagination of the plasma membrane in the anterior part of the cell where the flagellum emerges (Webster and Russell 1993; de Souza 2002) (Figure VI). Due to an adhesion zone between the plasma and flagellar membranes, the pocket generates as a secluded extracellular compartment. Although a dense subpellicular microtubule network extends along the whole cytoplasmic face of the plasma membrane (Gull 1999), this is absent in the area of the pocket, probably allowing transport and endocytic vesicle fusions (Landfear and Ignatushchenko 2001).

The proteins from the well developed endoplasmic reticulum and golgi, travelling through secretory vesicles, continue their journey to the flagellar pocket (Landfear and Ignatushchenko 2001; Souza 2006). Vesicles containing for example surface proteins fuse with the membrane in this region that connects to the plasma membrane. Other proteins are released into the lumen of the pocket where they either stay (Souto-Padron, Campetella et al. 1990; Duboise, Vannier-Santos et al. 1994; Monteiro, Abrahamson et al. 2001) or continue to internal organelles through the secretory pathway.

The **cytosotome** was first described in *Trypanosoma cruzi* (Milder and Deane 1969) as an opening of the plasma membrane close to the base of the flagella and flagellar pocket (Figure VI). It is connected to the flagellar pocket through a funnel-shaped structure formed by a deep invagination of the plasma membrane (de Souza 2002). The cytostome acts in a similar manner to the flagellar pocket, and in some trypanosomatids, it is actually the entrance to the pocket (Webster and Russell 1993), except in *Trypanosoma cruzi* epimastigotes (Vatarunakamura, Ueda-Nakamura et al. 2005).

**Figure VI: The Flagellar pocket and Cytostome of *Trypanosoma cruzi*.**

**Surface views of *T. cruzi* Y strain by High Resolution Field Emission Scanning Electron Microscopy.** (a): Epimastigote forms showed a smooth surface; (b) and (c) show the cytostome, which appears as a rugous and well delineated region, with an opening (arrow). (F) flagellum; (C) cytostome; (FP) flagellar pocket. Bars = 1  $\mu\text{m}$  (A); 100 nm (b–e)  
(Vatarunakamura, Ueda-Nakamura et al. 2005)  
© Blackwell Publishers Ltd.



### 3.1.3 The Reservosome

Reservosomes are endocytic organelles that are only found in the epimastigote stage of *Trypanosoma cruzi* (Soares and De Souza 1988; Sant'Anna, de Souza et al. 2004). This organelle is present in several copies, mainly in the posterior part of the cell (Sant'Anna, de Souza et al. 2004).

The name reservosome comes from the fact that this compartment accumulates all the macromolecules (proteins and lipids) ingested by the endocytic pathway and stores them for future use (Soares and de Souza 1991; de Souza 2002).

In *T. cruzi* epimastigotes in vitro, it has been shown that, under nutritional stress, the reservosomes disappear. This also occurs when the epimastigotes differentiate to metacyclic trypomastigotes (Figueiredo, Rosa et al. 2000; Figueiredo, Rosa et al. 2004); a process shown to be triggered by fluctuations in nutrient availability in the medium. This is another characteristic way for some trypanosomes to compartmentalize and store the energy resources, in order to rapidly adapt to environmental changes.

### 3.1.4 GPI-anchored proteins and Antigenic variation

Glycosylphosphatidylinositol (GPI) membrane anchors have been described in several organisms, e.g. bacteria, protozoa, yeast and humans. They are present in over than 200 eukaryotic cell-surface proteins (Ferguson 1999). The GPI anchors consist of carbohydrates, lipids, phosphates and amines, bound together in various combinations and conformations. They can be classified into three types: **type-I** containing the motif Man $\alpha$ 1-6Man $\alpha$ 1-4GlcN $\alpha$ 1-6PI; **type-II** is based on Man $\alpha$ 1-3Man $\alpha$ 1-4GlcN $\alpha$ 1-6PI; and the **hybrid type** with Man $\alpha$ 1-6(Man $\alpha$ 1-3) Man $\alpha$ 1-4GlcN $\alpha$ 1-6PI. Type II and hybrid GPIs are most abundant in trypanosomatids, which also express abundant glycoinositol phospholipids (GPIs) not attached to proteins on their cell surface (Ferguson 1999).

The contribution of trypanosomes to the research on GPI anchors was significant, due to the discovery of the variant surface proteins (VSGs) of African trypanosomes; proteins covering the whole cell surface of the parasite

and attached to the plasma membrane by covalent linkage to a GPI. The surface coat of *Trypanosoma brucei* (Vickerman and Luckins 1969) and its role in antigenic variation (Boothroyd 1985) promoted extensive research in this field, which contributed to the general knowledge of GPI biology.

Both *Trypanosoma cruzi* and *T. brucei* possess a dense arrangement of GPI anchored surface molecules, that help in the attachment of the parasite to different tissues and cells and protects them from the action of the immune system (Boothroyd 1985; Cross 1996). The genes coding for these proteins are present in the genome in high copy number (around 1000 copies of VSGs in *T. brucei* (Donelson 2003)) where the copies vary in sequence. This heterogeneity among the gene copies allows the parasite to express different versions of the same protein at different times (Borst, Bitter et al. 1998; Taylor and Rudenko 2006) during their life cycle; in other words switching exposed surface antigens and evading the immune system. This repositioning of the coat coincides with the waves of parasitemia detected in blood during phase 2 of African sleeping sickness (Section 2.2.2).

### **3.1.5 Polycistronic transcription and Trans-splicing**

A characteristic molecular feature of Trypanosomatidae is the regulation of gene expression. Normally eukaryote organisms regulate the transcription of their genes through transcription factors. These elements bind to the upstream RNA pol II-dependent promoter regions of genes and up- and down-regulate the production of messenger RNAs (mRNAs). This regulation process would be expected for these protozoans that constantly adapt to different conditions through their life cycle, but this is not the case (Clayton 2002). There is an almost complete absence of this type regulation.

Firstly, RNA pol II promoters are almost absent in trypanosomes and the protein-coding genes are not transcribed as single units, but as a polycistronic mRNA. Functionally unrelated genes can be part of the same transcription unit (Vijayasathy, Ernest et al. 1990; Campbell, Thomas et al. 2003; Manning-Cela, Jaishankar et al. 2006).

Secondly, mature and processed 5'-capped mRNAs are generated after a trans-splicing process where a splice leader (SL) RNA is added to each transcript to render an individual message (Ullu, Matthews et al. 1993; Gilinger and Bellofatto 2001). These splice leader mRNAs, also known as miniexons, are synthesized through RNA pol II transcription and the corresponding genes do have a RNA-pol II promoter (Campbell, Thomas et al. 2003; Ruan, Arhin et al. 2004). These transcripts are small capped RNAs of approximately 140 bases but only the first 39 nucleotides are ligated into the final mRNA (Ullu, Matthews et al. 1993).

### **3.1.6 The Acidocalcisome and Contractile vacuole**

The acidocalcisome was first described in trypanosomes (Vercesi, Moreno et al. 1994; Docampo, Scott et al. 1995) and was much later on discovered to be present in several other organisms from bacteria (volutin

granules) to humans (dense granules in platelets) (Seufferheld, Vieira et al. 2003; Ruiz, Lea et al. 2004; Docampo, de Souza et al. 2005). These organelles are present in varying numbers per cell and up to 40 acidocalcisomes can be visualized in *T. cruzi* epimastigotes (Miranda, Benchimol et al. 2000).

The name acidocalcisome comes from the acidic character of its lumen and the high calcium content (Docampo, Scott et al. 1995). This organelle also contains traces of amino acids, P, Mg, Na, Zn, Fe, and very little Cl, K, and S (Scott, Docampo et al. 1997; Miranda, Benchimol et al. 2000). The low content of sulfur (S) suggests low protein content. Phosphorous in the form of pyrophosphate (PPi) and short- and long-chain polyphosphates (PolyP) are also present (Docampo and Moreno 1999; Urbina, Moreno et al. 1999).

Only a few proteins have been localized to the matrix of the acidocalcisomes. In the lumen, an exopolyphosphatase (PPX) (Rodrigues, Ruiz et al. 2002), a polyphosphate kinase (PPK) (Ruiz, Rodrigues et al. 2001) and a soluble inorganic pyrophosphatase (PPase) (Lemercier, Espiau et al. 2004) involved in the phosphate metabolism in the acidocalcisome are present (Moreno, Urbina et al. 2000; de Souza 2002; Docampo, de Souza et al. 2005).

On the other hand, several pumps and exchangers have been found in the membrane of the organelle:

- A vacuolar H<sup>+</sup>-ATPase involved in acidification (Docampo, Scott et al. 1995)
- A vacuolar H<sup>+</sup> Pyrophosphatase (H<sup>+</sup>-PPase) (Scott, de Souza et al. 1998; Lemercier, Espiau et al. 2004)
- A Ca<sup>2+</sup>-ATPase involved in calcium uptake (Vercesi, Moreno et al. 1994; Docampo, Scott et al. 1995)
- A Na<sup>+</sup>-H<sup>+</sup> exchanger involved in pH control or acidification (Vercesi and Docampo 1996)
- A Ca<sup>2+</sup> -H<sup>+</sup> exchanger (Vercesi, Grijalba et al. 1997)
- An aquaporin, a water channel involved in cell osmoregulation (Montalvetti, Rohloff et al. 2004)
- A vacuolar transporter chaperone (VTC) essential for poly P synthesis, acidocalcisome biogenesis and cytokinesis (Fang, Rohloff et al. 2007).

Due to the presence of these pumps and exchangers in the acidocalcisome and its particular content, especially calcium and PolyP, several essential functions have been proposed for this organelle.

**1) Calcium storage.** Ca<sup>2+</sup> is involved in several signaling processes shown to be important for the cells. Calcium enters cells through the plasma membrane, but in the case of intracellular parasites such as *T. cruzi* calcium availability is limited. A way to overcome this matter is to store calcium and release it into the cytoplasm when needed. This is the case especially during host cell invasion of the parasite, where soon after the parasite internalization there is an increase of the parasite cytoplasmic Ca<sup>2+</sup> concentration (Moreno, Silva et al. 1994; Docampo and Moreno 1999).

**2) pH homeostasis.** The acidic property of the acidocalcisomes and the presence of a H<sup>+</sup>-ATPase and a H<sup>+</sup> PPase in the organelle membrane, infer a role in maintenance of the pH balance of the cell. Hydrolysis of the

Poly P inside the acidocalcisomes may be a source of protons (H<sup>+</sup>) for this matter (Lemerrier, Dutoya et al. 2002; Docampo, de Souza et al. 2005).

**3) An energy store organelle.** The large amount of inorganic PPi and polyphosphates suggests a role in the bioenergetics of the cell. Poly P are linear chains of inorganic phosphates linked together by high-energy phosphoanhydride bonds (Kornberg, Rao et al. 1999; Docampo, de Souza et al. 2005).

The polyphosphate kinase (PPK) synthesizes poly P from ATP in a reversible reaction that also converts ADP back to ATP and shortens poly P chains (Kornberg, Rao et al. 1999). **[nATP ↔ poly P<sub>n</sub> + nADP]**

In other words, these polymers are a storage-source of ATP for the cell. Poly P may also act as an ATP substitute for kinases and as a Pi donor for sugars and proteins.

Accumulation or hydrolysis of poly P varies when cells are either under nutritional stress (depletion in Pi, amino acids, nitrogen and nutrients in general), and osmotic changes; or during cell growth and differentiation linked to calcium signaling (Ruiz, Rodrigues et al. 2001).

**4) Osmoregulation.** The acidocalcisomes also play a role in cellular osmoregulation (Docampo and Moreno 1999). Poly P synthesis-hydrolysis responds to changes in osmolarity, accumulating with higher salt levels and hydrolyzing when under hypo-osmotic stress (Ruiz, Rodrigues et al. 2001).

Another way for trypanosomes to control the intracellular environment for adaptation is through the flux of water. An aquaporin has been located to the acidocalcisome of *Trypanosoma cruzi* (Montalvetti, Rohloff et al. 2004) and to the **contractile vacuole** complex, and both organelles have been shown to play a role in osmoregulation (Rohloff, Montalvetti et al. 2004).

The **contractile vacuole complex** (CVC) is composed of a two-compartment system enclosed by two differentiated membranes, often divided into numerous vesicles and tubules (Gerisch, Heuser et al. 2002). It contains many proton-translocating V-H<sup>+</sup>-ATPase enzymes that provide an electrochemical gradient of protons for water transport (Allen and Naitoh 2002; Rohloff, Montalvetti et al. 2004).

### 3.1.7 Glycosomes - Redox & Energy balance in Carbohydrate metabolism

In trypanosomatids, most of the glycolysis pathway enzymes are located in the **glycosome**. The Glycosomes (Opperdoes and Borst 1977) are microbody-type or peroxisome-like organelles that distribute along the cell in varying numbers between stages and strains (de Souza 2002).

The glycosomes contains the glycolysis pathway (Opperdoes and Borst 1977; Visser and Opperdoes 1980) and the carbohydrate metabolism, as well as several other typical peroxisomal enzymes belonging to the purine-pyrimidine pathways, sterol metabolism, β-oxidation of fatty acids, ether-lipid biosynthesis, and other enzymes thought to contribute to maintain the energy



and redox balance inside the organelle (Michels, Hannaert et al. 2000). Glycosomes differ from eukaryote peroxisomes by the absence of catalase, the alkalinity of the enzymes in the lumen, and the presence of pathways that are normally present in the cytosol of other eukaryotes.

Glycosomes are poorly permeable to most metabolites. Therefore, all substrates and products inside the organelle must be in a stoichiometric balance at all times (Hannaert, Bringaud et al. 2003). Compartmentalization also keeps the glycosomal and cytoplasmic pools of substrates and products (ATP:ADP and NAD<sup>+</sup>:NADH) controlled and maintains a basal ATP level that will allow rapid glucose utilization following periods of starvation (Bakker, Mensonides et al. 2000; Michels, Hannaert et al. 2000). Inorganic pyrophosphate (PPi) is also produced inside the glycosomes as a byproduct that needs to be hydrolyzed to avoid reaching levels that are toxic for the glycolytic enzymes. A pyruvate phosphate dikinase (PPi-dependent) (PPDK) (Bringaud, Baltz et al. 1998) was suggested to be responsible for controlling these levels. This is one more characteristic adaptation to be able to rapidly respond when switching between different environments.

## 4 CHEMOTHERAPEUTICS for CHAGAS DISEASE

As discussed in Section 2.1.6, there is no cure for Chagas disease or for trypanosomiasis in general. Besides Nifurtimox and Benznidazole, the only two drugs available against *T. cruzi*, many other compounds have been tested on chagasic patients since the 1970's. Although some generated promising results, the side effects were also severe. Many compounds and drugs in use for treating other malignancies have been added to the list of tested compounds and many more are being tested (Nwaka and Hudson 2006).

The complete process from evaluating a compound as a drug candidate to its approval to be tested in humans and its availability on the market takes many years and much funding. As Chagas disease and other parasitic infections affect mainly poor areas, it is not profitable for private pharmaceutical companies to invest in these malignancies (Witty 1999). In the view of this, drug discovery in tropical diseases relies on the extension of the use of existing parasitic drugs to treat others; using drugs for other malignancies, with known molecular targets, and redesign them for another disease (Liendo, Lazard et al. 1998; Witty 1999); *de novo* drug discovery through high throughput screening (HTS) of registered drug libraries (Holloway, Baell et al. 2007), and *in silico* screening of genomes, and structural data (Nwaka and Hudson 2006; Woods and Williams 2007; Sayed, Simeonov et al. 2008; Simeonov, Jadhav et al. 2008).

All these approaches require improved knowledge of the molecular biology of the parasites, and this is improving rapidly thanks to academic research.

### 4.1 METABOLIC PATHWAYS and PROTEINS under DRUG TARGET EVALUATION

Some of the potential target metabolic pathways and key enzymes under evaluation for drug design against *Trypanosoma cruzi*, and trypanosomatids in general, are discussed below.

#### 4.1.1 Polyamine biosynthesis

Polyamines are low-molecular-weight compounds that are essential for cellular processes such as nucleic acid and protein synthesis and stabilization of certain macromolecules (Bacchi, Nathan et al. 1980). The common polyamines are putrescine, spermidine, and spermine. Several enzymes are involved in polyamine synthesis: arginase, ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, trypanothione synthetase (TS), and trypanothione reductase (TR). These enzymes exhibit features that differ from those of the human host, and certain enzymes that use polyamines as substrates are unique to the parasites (Heby, Persson et al. 2007). Inhibitors affecting this pathway are as a consequence promising targets for drug design (Bacchi,

Nathan et al. 1980; McCann, Bacchi et al. 1988; Bacchi, Brun et al. 1996; Taylor, Kaur et al. 2008).

#### **4.1.2 Antioxidant metabolism and Trypanothione reductase (TR)**

Trypanothione reductase, apart from being part of the polyamine pathway, is involved in the antioxidant metabolism in trypanosomes. TR is a flavoenzyme that keeps trypanothione in its reduced form and available for oxidation by trypanothione oxidase. This maintains an intracellular reducing environment and prevents oxidative stress. The trypanothione/trypanothione reductase system recognizes a different substrate than its mammalian counterpart, the glutathione/glutathione reductase (GR) system, which makes it a promising target for drug design (Schmidt and Krauth-Siegel 2002; Czechowicz, Wilhelm et al. 2007). Nitrofuranes have been found to inhibit trypanothione reductase (Maya, Bollo et al. 2003; Aguirre, Cabrera et al. 2004).

#### **4.1.3 Proteases - Cystein protease**

Cruzipain is a cystein protease and a member of the papain C1 family. The mature *T. cruzi* proteinase has a catalytic moiety that is homologous to cathepsins S and L, that is absent from all the other family members (Cazzulo, Stoka et al. 1997; Jose Cazzulo, Stoka et al. 2001). Cruzipain, also known as cruzain, is a multicopy gene in the genome of *T. cruzi*. It is expressed as a mixture of isoforms and is the most abundant protease in the parasite (Cazzulo 2002). This protease has shown to be important in the host-parasite relationship (Santos, Sant'anna et al. 2005) and inhibitors of cruzipain kill the parasite and cure infected mice (Meirelles, Juliano et al. 1992; Franke de Cazzulo, Martinez et al. 1994; Barr, Warner et al. 2005; Doyle, Zhou et al. 2007). It is a very promising target for drug development in Chagas disease.

#### **4.1.4 Purine synthesis - Hypoxanthine-guanine phosphoribosyltransferase**

Trypanosomatids do not synthesize purines de novo, they rely on the host recycled nucleotides and their purine salvage pathway (Berens, Marr et al. 1981). Trypanosomes convert purine bases to ribonucleotides by the action of the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) that makes use of hypoxanthine, which is highly abundant in human serum (Eakin, Guerra et al. 1997).

This enzyme is crucial for parasite survival. Purine analogs that inhibit HGPRT interfere with trypanosome cell proliferation inside the host. One of the most tested analogs against *Trypanosome cruzi*, is allopurinol (Aloprim®, Zylprim®) (Berens, Marr et al. 1982), which is available in the market for treatment of gout, high levels of uric acid in the body caused by certain cancer medications, and kidney stones. But the effects of this drug for the treatment of Chagas disease are not yet clear (Gobbi, Lo Presti et al. 2007).

#### 4.1.5 GPI-biosynthetic pathway

The glycosylphosphatidylinositol (GPI) synthesis pathway could be a target for drug development. Cell-membrane-permeable GlcN-PI and GlcNAc-PI analogs were found to inhibit the GPI synthesis (Smith, Crossman et al. 2004). The accumulation of the metabolites generated altered the pathway and showed trypanocidal effects. As the human GPI pathway is not affected in the same way, GPI synthesis inhibitors are under evaluation as drugs.

#### 4.1.6 Sterol Biosynthetic Pathway - Polyprenyl synthases

Sterols are important in all organisms as they have structural functions in cellular membranes and are involved in other processes, such as cell cycle control (Ginger, Prescott et al. 2000). *Trypanosoma cruzi* requires endogenous sterols for cell proliferation and viability (Liendo, Lizardi et al. 1998).

While cholesterol is the main sterol detected in mammalian membranes, the main sterol in *T. cruzi* is ergosterol. This is also the case for fungi (Demel and De Kruffy 1976). This difference from mammals regarding sterol composition and sterol biosynthesis, has directed attention to the sterol pathway as a target for drugs against trypanosomiasis and especially to Chagas disease (Urbina, Lizardi et al. 1988; Urbina 1997; Roberts, McLeod et al. 2003). In addition, pharmaceutical companies have developed a wide range of antifungal drugs that target the sterol biosynthetic pathway, with no toxicity for humans.

Among the sterol biosynthesis inhibitors (SBIs) tested in trypanosomatids are allylamines, imidazoles and triazoles compounds and azasterols (Urbina, Lizardi et al. 1988; Goad, Berens et al. 1989; Maldonado, Molina et al. 1993; Roberts, McLeod et al. 2003). These compounds target different enzymes in the biosynthesis pathway:

3-hydroxy-3-methylglutaryl CoA reductase (HMGR) (Hurtado-Guerrero, Pena-Diaz et al. 2002)

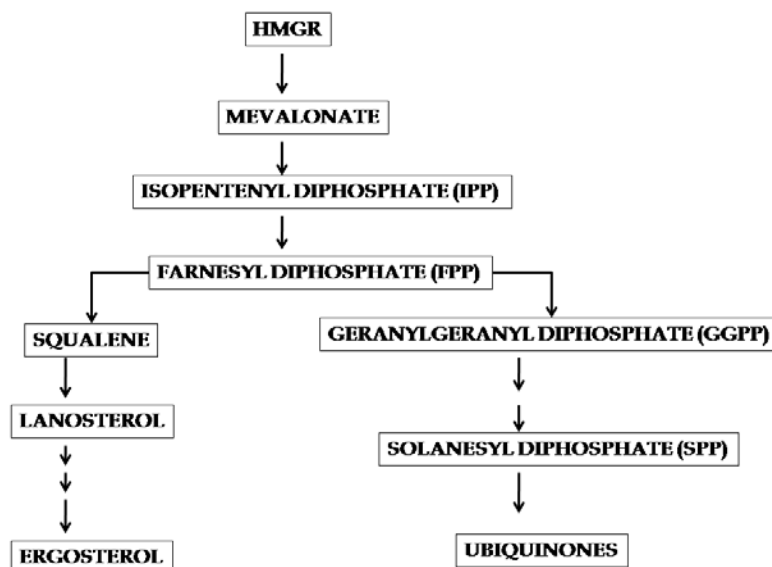
Squalene synthase (SQS)(Braga, Urbina et al. 2004)

Sterol C14-demethylase (Buckner, Yokoyama et al. 2003; Corrales, Cardozo et al. 2005; Hankins, Gillespie et al. 2005)

24-sterol methyltransferase (Urbina, Vivas et al. 1996; Gros, Castillo-Acosta et al. 2006)

Lanosterol synthase (E.C. 5.4.99.7, oxidosqualene cyclase, OSC) (Buckner, Griffin et al. 2001; Galli, Oliaro-Bosso et al. 2007)

**Scheme I: Simplified partial sterol biosynthesis pathway.** Precursors and intermediates of the sterol metabolism discussed along this thesis are represented in this scheme.



Other target enzymes under evaluation for drug design that are part of this pathway are polyprenyl diphosphate synthases (PPS) and protein farnesyltransferases (PFT) (Ohkanda, Buckner et al. 2004; Hucke, Gelb et al. 2005). These enzymes are involved in the synthesis of essential linear isoprenoids and their transfer to signaling proteins, respectively.

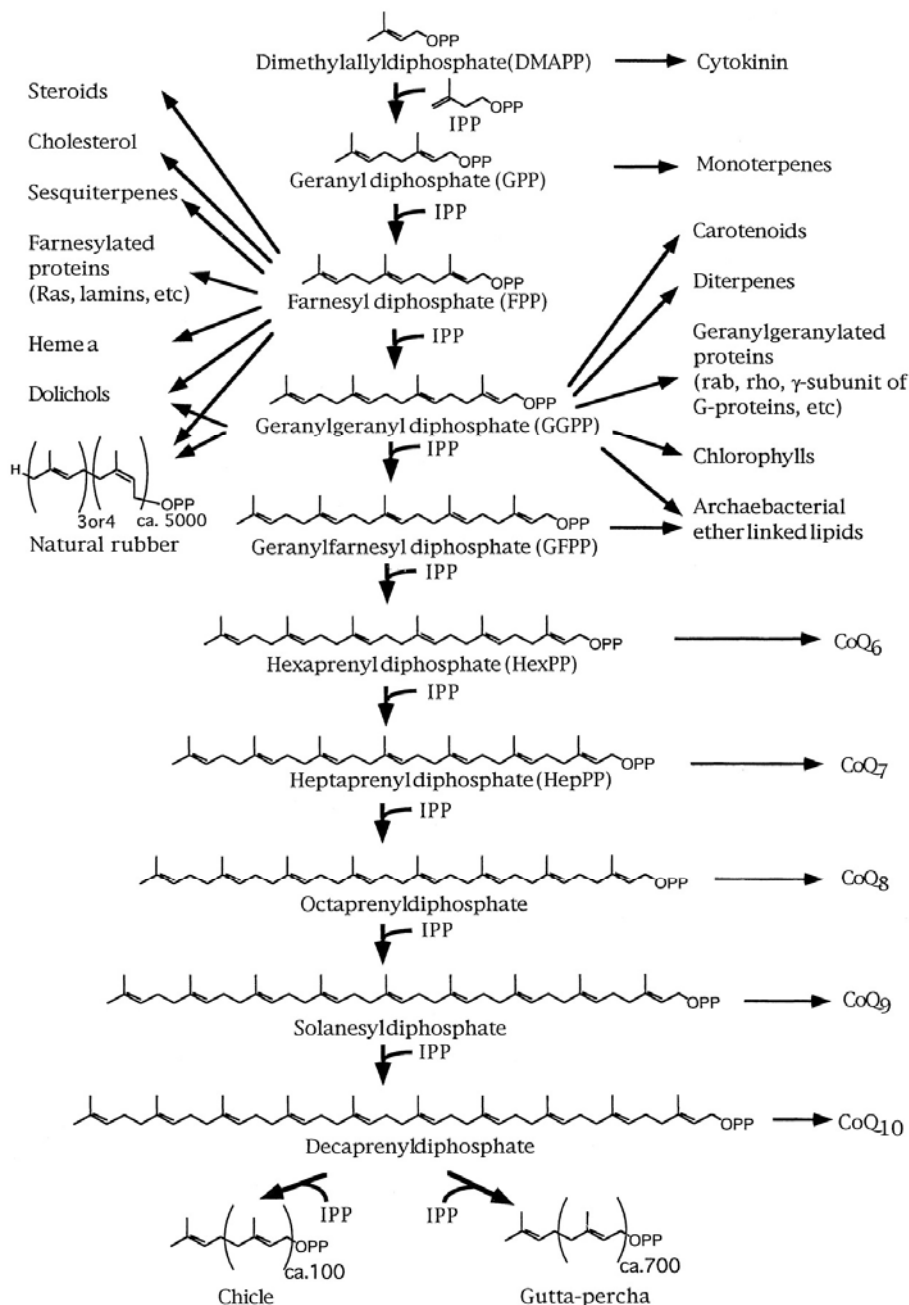
Of particular interest are the results obtained from the inhibition of farnesyl diphosphate synthases by nitrogen containing biphosphonates otherwise used for the treatment of osteoporosis and other bone related malignancies (Martin, Arnold et al. 1999; Montalvetti, Bailey et al. 2001; Montalvetti, Fernandez et al. 2003; Szajnman, Montalvetti et al. 2003). Different biphosphonate derivatives have also proven to be effective inhibitors of the solanesyl diphosphate synthase (SPPS) (Szajnman, Garci et al. 2007) in *T. cruzi*.

The selective action of these nitrogen biphosphonates against *T. cruzi* compared to mammalian cells could result from the accumulation of the drug inside acidocalcisomes, which contain high levels of phosphates (Urbina, Moreno et al. 1999; Docampo and Moreno 2001) (discussed previously in Section 3). The soluble vacuolar pyrophosphatase from *T. brucei* (TbVSP1) which is not present in humans, was also inhibited by biphosphonates (Kotsikorou, Song et al. 2005).

## 5 ISOPRENOID BIOSYNTHESIS PATHWAY

Isoprenoids are the most abundant naturally occurring compounds, with over 20,000 members. Some of the most important molecules are sterols, ubiquinones, dolichols, carotenoids, prenyl groups and plant terpenes (Chen, Kroon et al. 1994), which are all derived from the linear isoprenoids synthesized by polyprenyl synthases (Figure VII).

**Figure VII: Isoprenoid biosynthesis pathway.** IPP, synthesized from the mevalonate pathway, is condensed with DMAPP to start the isoprenoid biosynthetic series of condensations, to produce all the intermediates compounds to be used in other biosynthesis pathways (Ohnuma, Hirooka et al. 1998). © JBC - (Reproduced with permission).

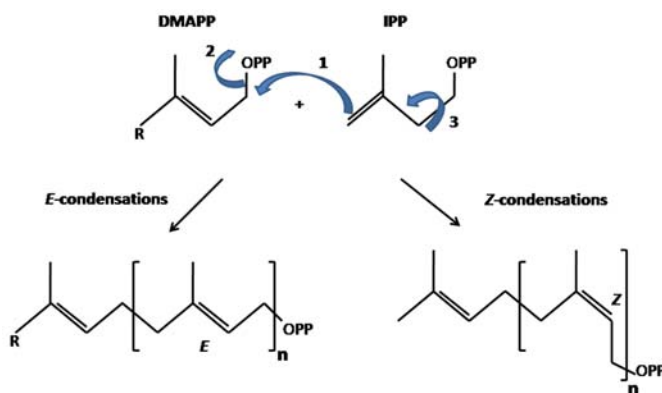


Protein prenylation has been demonstrated in parasites: *Giardia lamblia* (Lujan, Mowatt et al. 1995); *Schistosoma mansoni* (Chen and Bennett 1993); *T. brucei* (Field, Blench et al. 1996; Yokoyama, Lin et al. 1997); *T. cruzi* and *Leishmania mexicana* (Yokoyama, Trobridge et al. 1998); *Toxoplasma gondii* (Ibrahim, Azzouz et al. 2001), and *Plasmodium falciparum* (Chakrabarti, Azam et al. 1998; Chakrabarti, Da Silva et al. 2002). This modification is of importance for signaling proteins and receptors involved in cell proliferation and survival.

Ubiquinones, are not only essential in mitochondrial membranes for cellular respiration, but they also function as lipid soluble antioxidants (Frei, Kim et al. 1990) and are members of sulfide metabolism (Szkopinska 2000), probably coupled to trypanothione balance (Watanabe, Dickinson et al. 2004). Organisms in nature possess ubiquinones (UQ) with different length isoprenoid side chains, as determined by the polyprenyl diphosphate synthases.

## 5.1 POLYPRENYL DIPHOSPHATE SYNTHASES

Polyprenyl diphosphate synthases, also called isoprenyl diphosphate synthases, are enzymes that catalyze the sequential condensation of isopentenyl pyrophosphate (IPP, C<sub>5</sub>) with allylic substrates of five carbon units, to produce linear isoprenoids of variable lengths. The reaction is an irreversible 1'-4 head-to-tail condensation; a bimolecular nucleophilic substitution (S<sub>N</sub>2) (Cornforth, Cornforth et al. 1966; Barnard 1985) (Scheme II).



**Scheme II: 1'-4 head-to-tail condensation.** (1) The carbon at position 1 (C1) from IPP acts as a nucleophile and attacks the C4-OPP bond from DMAPP. C4 then condense with C1; (2) the OPP group is liberated; (3) a proton is removed from the IPP carbon 3, and a double bond is formed with C2.

**Nucleophile:** literally *nucleus lover*. All molecules or ions with two free electrons can act as nucleophiles.

The polyprenyl synthases are classified according to the stereochemistry of the double bond formed by the condensation reaction into *Z*- or *cis*-polyprenyl diphosphate synthases and *E*- or *trans*-polyprenyl diphosphate synthases (Koyama 1999; Liang, Ko et al. 2002).

A second classification is based on the prenyl chain length of the final product they synthesize (Koyama 1999). Each enzyme terminates the elongation of the linear isoprenoid at a definite chain length according to their particular specificity (Table II).

**Table II: Summary of the polyprenyl synthases, the respective preferred allylic substrate and the maximum length isoprenoid synthesized.**

|                         | <b>Polyprenyl Synthase (PPS)</b> | <b>Allylic Substrate</b> | <b>Final Product</b>   |
|-------------------------|----------------------------------|--------------------------|------------------------|
| <b>Short chain PPS</b>  | GPPS                             | DMAPP                    | GPP, C <sub>10</sub>   |
|                         | FPPS                             | DMAPP/GPP                | FPP, C <sub>15</sub>   |
|                         | GGPPS                            | GPP/FPP                  | GGPP, C <sub>20</sub>  |
| <b>Medium chain PPS</b> | GFPPS                            | FPP                      | GFPP, C <sub>25</sub>  |
|                         | HexPPS                           | FPP                      | HexPP, C <sub>30</sub> |
|                         | HepPPS                           | FPP                      | HepPP, C <sub>35</sub> |
| <b>Long chain</b>       | OPPS                             | FPP/GGPP                 | OPP, C <sub>40</sub>   |
|                         | SPPS                             | FPP/GGPP                 | SPP, C <sub>45</sub>   |
|                         | DPPS                             | FPP/GGPP                 | DPP, C <sub>50</sub>   |
|                         | UPPS                             | FPP/GGPP                 | UPP, C <sub>55</sub>   |

G: geranyl; F: farnesyl; GG: geranylgeranyl; GF: geranylarnesyl; Hex: hexaprenyl; Hep: heptaprenyl; O: octaprenyl; S: solanesyl; D: decaprenyl; and U: undecaprenyl.

Short chain polyprenyl synthases (geranyl, farnesyl and geranylgeranyl diphosphate synthases) catalyze the production of short chain prenyl diphosphates (GPP, C<sub>10</sub>; FPP, C<sub>15</sub>; and GGPP, C<sub>20</sub>, respectively). These enzymes require magnesium or manganese ions and they are homodimers (except for GPPS).

Medium chain polyprenyl synthases are hexaprenyl (HexPPS) and heptaprenyl (HepPPS) diphosphate synthases that produce (all-*E*)-hexaprenyl and (all-*E*)-heptaprenyl diphosphates, respectively. These are side chains of ubiquinones in some organisms. They differ from the other polyprenyl synthases in that they have a heteromeric structure. Medium chain enzymes are formed by two distinct easily dissociable components. The larger subunit contains the conserved motifs of the polyprenyl synthases while the smaller subunit has little sequence similarity to this family. The subunits associate in the presence of the allylic substrate (FPP) and magnesium (Hirooka, Ohnuma et al. 2000; Zhang, Li et al. 2000).

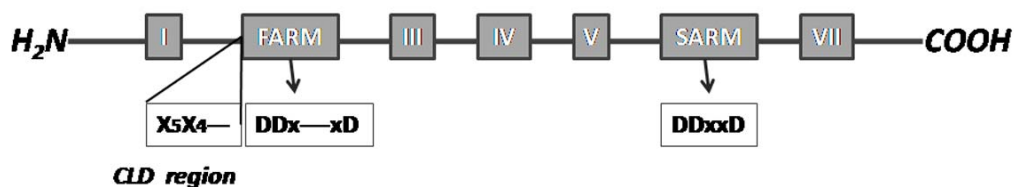
*Cis*- and *trans*-long chain prenyl diphosphate synthases are, homodimers that produce linear isoprenoids ranging from C<sub>40</sub> (side chain of ubiquinones, Q<sub>8</sub>-Q<sub>10</sub>) to C<sub>110</sub>. They require additional factors that are involved in removing the hydrophobic isoprenoid from the active site (Koyama 1999).

### **5.1.1 Sequence conservation among all-*E*-polyprenyl diphosphate synthases**

All-*E*-polyprenyl synthases are very well conserved enzymes with 5 to 7 conserved domains (Scheme III). Two of them, the first and second aspartate rich motifs (FARM and SARM), are involved in binding the IPP and allylic substrate, respectively (Chen, Kroon et al. 1994; Wang and Ohnuma 1999; Liang, Ko et al. 2002).



The Z-type synthases show a different structure and conserved motifs, although an aspartate rich region similar to FARM has been identified (Shimizu, Koyama et al. 1998; Koyama 1999).



**Scheme III: Schematic representation of a protein bearing the seven conserved motifs of the polyprenyl synthase family.** FARM (first aspartate rich motif) with the conserved aspartate (D) residues (DDx---xD); SARM (second aspartate rich motif) and conserved D residues (DDxxD). X or x denotes any other amino acid; --- denotes variable amino acid sequence length. CLD (chain length determination) region shows the -5 and -4 amino acid positions to the FARM.

The aspartate rich motifs are also known as polyprenyl synthase signature 1 and 2 (<http://ca.expasy.org/cgi-bin/prosite>):

- (1) **PS00723:** [LIVM](2)-x-D-D-x(2,4)-D-x(4)-R-R-[GH];
- (2) **PS00444:** [LIVMFY]-G-x(2)-[FYI]-Q-[LIVM]-x-D-D-[LIVMFY]-x-[DNG].

Mutational studies of the amino acids surrounding the FARM and SARM showed that several amino acids are involved in determining the specific final product length generated by each enzyme. The amino acids located at positions -5 and -4 to FARM were particularly important (the CLD region) (Wang and Ohnuma 1999; Liang, Ko et al. 2002; Guo, Kuo et al. 2004). Mutations near the SARM also affected the chain length of the synthesized isoprenoid (Hemmi, Noike et al. 2003). In the case of medium chain enzymes, where only one subunit contains the PPS conserved regions, the amino acids in the FARM of component II are involved in specificity (Hirooka, Ohnuma et al. 2000). This is conserved among all-*trans*-polyprenyl synthases.

When the crystal structure for the avian FPPS was reported (Tarshis, Yan et al. 1994), it confirmed a protein 3D structure dominated by alpha helices as predicted by hydrophobicity profiles (Chen, Kroon et al. 1994). There is an arrangement of ten alpha helices around a large central cavity, where the two aspartate-rich regions were found on opposite walls. The catalytic site is located in the cavity and the aspartate residues mediate the binding of the homologous subunits via magnesium ion (Mg<sup>2+</sup>) bridges (Tarshis, Yan et al. 1994).

Crystallography also showed that the amino acids involved in the determination of the prenyl chain final length were located at specific turns of the helices. They interfere with the extension of the isoprenoid in the case of a bulky amino acid, and allows a longer chain isoprenoid in case of a small residue (Tarshis, Yan et al. 1994; Guo, Kuo et al. 2004).

## 6 GENE-PROTEIN IDENTIFICATIONS

Approaches to identify novel proteins in different species, including trypanosomatids, for many years involved the detection of an endogenous activity or the function of a protein by biochemical methods followed by the purification and characterization of the protein. This strategy required several steps, including chromatography, dialysis for buffer exchange and activity assays to follow up the purification.

In the 1970s and 1980s, when recombinant DNA technology and PCR became available (Saiki, Scharf et al. 1985; Saiki, Gelfand et al. 1988; Westerhoff and Palsson 2004), gene discovery became easier. Databases of DNA sequences, such as EST / cDNA databases, were built from RNA isolation from several species, especially model organisms.

In the case of *Trypanosoma cruzi*, several libraries were created to map the genome in the 1990s: EST (Brandao, Urmenyi et al. 1997), cosmid (Hanke, Sanchez et al. 1996), YAC (yeast artificial chromosome) and BAC (bacterial artificial chromosome) libraries (Ferrari, Lorenzi et al. 1997). These efforts were coordinated by the WHO funded *T. cruzi* Genome Network, with participants from many countries. The information available from partially sequencing these libraries and annotating the sequences using homology to other species was useful for the design of the functional studies of the detected genes.

Sequence comparisons between species became a way to putatively identify a gene product. The use of sequence alignment tools, hydrophobicity profile comparisons and transmembrane and signal peptide predictions in many cases provided initial information of the putative location and function of the genes.

With the availability of whole-genome sequences [mid-1990s] and bioinformatic tools allowing comparative studies (Joyce and Palsson 2006) the amount of data available from different organisms increased enormously. Since 1995 almost 300 genome sequencing projects have been completed and more are on the way (<http://www.genomesonline.org/>). Genome annotations were performed using homology analysis between species, nucleotide and protein sequence comparisons, computational tools detecting patterns resulting in functional domains, and others.

However, when genome comparisons fail due to low sequence homology and conservation among compared organisms, direct methods for protein identification by functional studies are needed.

Single genes or single pathways are studied through standard biochemical and molecular techniques. Some of these methods and tools are, in general terms: PCR (traditional, quantitative, real time), cloning vectors, restriction enzymes, heterologous expression in bacteria and yeast, antibody production, fluorescent, confocal and electron microscopy, southern and northern blots for DNA and RNA analysis, enzymatic activities, systems for detecting protein-protein (e.g.: two-hybrid system; FRET; cross-linking) and protein-DNA interactions (gel shift assays), RNA interference (RNAi) and electrophoresis methods for protein resolution, among many others.

High-through-put analysis of RNA, protein and other features followed from the genome era, and are now known as 'omics' technologies. These technologies make use of the standard procedures mentioned above, in parallel for the study of hundreds to thousands of genes and proteins.

Transcriptomics is mostly based on microarrays (Hardiman 2004) and SAGE (serial analysis of gene expression) (Harbers and Carninci 2005) technology. SAGE involves a massive sequence analysis of short cDNA sequence tags derived from cellular or tissue RNA. Gene identification and expression levels can be achieved with both methods.

Proteomics, through the use of mass spectrometry, confronted a much more complex picture due to the vast number of modifications and multiple splicing variants of proteins in a particular genome. But from the initial limited MALDI techniques the field has developed making it possible to carry out whole cell proteomics.

RNA interference (RNAi), first described in *C. elegans* (Fire, Albertson et al. 1991), is widely used for silencing genes and thus for functional characterizations of a gene product. This technique has been successfully used in *T. brucei* but does not work in *T. cruzi* (DaRocha, Otsu et al. 2004; DaRocha, Silva et al. 2004).

Genomics, proteomics, RNA interference and all the other techniques included here are all used for rapid mapping of a gene sequence and the characterization of the corresponding protein(s) and their function(s).

## 7 TRYPANOSOMA CRUZI GENOME

### 7.1 PHYLOGENY

*Trypanosoma cruzi* is a heterogeneous species that comprises several populations circulating among wild and domesticated animals and humans. They differ in genetic content, biochemical processes, host tropism and infectivity (Andrade 1999).

The natural stocks of this parasites can be divided into two main lineages: *T. cruzi* I which is mostly related to the sylvatic cycle of the transmission and *T. cruzi* II, which is more often involved in the domestic cycle and severe Chagas disease (Vargas, Pedroso et al. 2004). Within *T. cruzi* II, there are currently 5 subdivisions (IIa to IIe) (Barnabe, Brisse et al. 2000; Brisse, Barnabe et al. 2000; Breniere, Barnabe et al. 2003) as classified using multilocus markers such as isoenzymes, RAPD (Random Amplification of Polymorphic DNA) and microsatellites (Zingales, Pereira et al. 1997; Oliveira, Broude et al. 1998).

Genetic exchange between natural stocks of *Trypanosoma cruzi* has been shown to occur (Gaunt, Yeo et al. 2003). The formation of the hybrid progeny genotype involves fusion of parental genotypes, loss of alleles, and aneuploidy. The *Trypanosoma cruzi* CL Brener strain (*T. cruzi* sub-group IIe), the reference strain for the genome sequencing project, is a hybrid from sub-clones of the lineage type II (Gaunt, Yeo et al. 2003), although there is evidence of satellites repeats from lineages type I and II (Elias, Vargas et al. 2005). It was chosen as the reference strain because of the extensive experimental characterization of the strain (El-Sayed, Myler et al. 2005) and it also met several desired characteristics of a model isolate (Cano, Gruber et al. 1995): it is derived from the domiciliary vector *Triatoma infestans*, differentiates well in the simple liver infusion tryptose (LIT) medium, is able to infect mammalian cells with preference of heart and muscle cells and causes mortality in mice.

### 7.2 GENOME PROJECT

In several protozoa including *T. cruzi* the chromatin is poorly condensed during mitosis (Henriksson, Aslund et al. 1996), which makes it difficult to determine the number of chromosomes, their sizes and the complete genome size. Also, strains, and even clones derived from the same strain differ in the total DNA content per cell (Santos, Cano et al. 1997).

The initial approach for the *T. cruzi* genome sequencing project focused on individual chromosomal bands that could be isolated by pulse field gel electrophoresis (PFGE) and hybridization of EST probes over these bands to map cosmid clones to the genome (Henriksson, Aslund et al. 1990; Cano, Gruber et al. 1995). Other information such as estimation of the DNA content and relative copy number of chromosomes in a band came from densitometry analysis of the bands in the pulsed field gels.

PFGE hybridization patterns showed that homologous chromosomes often had different sizes (Henriksson, Aslund et al. 1990), and that groups of markers always presented identical hybridization patterns, which indicated the presence of syntenic groups. These were conserved among different strains. The term “synteny” refers to the conservation of gene order.

In 2002 the genome project changed approach from mapping individual chromosomes using BAC clones to whole genome shotgun (WGS) (Deininger 1983; Roach 1995) sequencing, due to the repetitive nature of the genome. A genome assembly with ~7x coverage of the diploid genome, produced by three collaborating laboratories, was released in 2005 (El-Sayed, Myler et al. 2005).

*Trypanosoma cruzi* genome features:

- Assembly size: 67 Mb (megabases)
- Estimation of the total diploid genome: 106.4 to 110.7 Mb
- Presence of two different polymorphic haplotypes: 30.5 Mb present twice in the assembly.
  
- The non-hybrid Esmeraldo strain, typed as a member of the progenitor group IIb, was sequenced with a low coverage to help in the assembly of the CL Brener genome. It was used to separate the hybrid haplotypes by comparison to the Esmeraldo assembly.
  
- Genes per haploid genome: ~12,000
- Total estimated protein-coding genes in the diploid genome: 22,570
- Genes from haplotype IIb: 6159
- Alleles from the second haplotype: 6043
- Genes not assigned to any haplotype: 10368 (this number includes large families of surface proteins)
- Repetitive DNA: 50% of the genome is repetitive

Repetitive sequences and genes were detected in many regions, and the repeat content could be even higher than 50% due to tandem repeats that collapsed into fewer copies in the assembly. The various repetitive gene families in *T. cruzi* include the surface molecules trans-sialidase (TS), mucins, mucin associated proteins (MASP) and glycoproteins (gp63). 1430 members of the TS family; 863 mucins; 1377 MASPs and 420 genes and pseudogenes of gp63 family, were detected in the genome assembly (El-Sayed, Myler et al. 2005).

- Putative functions could only be assigned to 50.8% of the protein-coding genes, based on gene ontology (GO), Pfam domains and BLAST searches.

Thus, there are several thousand hypothetical proteins in the genome with unknown function and low or no homology to any species known to date. Post-genomic functional studies will have to be carried out in order to address their function.

## 8 PROTEOMICS

The complete set of proteins expressed in a cell, tissue or organism, make up the “proteome” of that cell, tissue or organism.

The proteome is much more complex than a genome. Several different proteins can be the product of a single gene due to post-transcriptional processing; sequence polymorphisms; post-translational modifications that can create a vast set of proteins and expression profiles. The protein repertoire can change in response to life cycle or environmental changes.

Proteomics was the term initially used to describe the large scale identification and quantitation of proteins encoded by a genome. Today, proteomics has been expanded to include any post-genomic study with the aim to identify gene products, to determine all protein isoforms and modifications, to determine interactions between proteins, to describe protein structures and structural complexes, to assess expression profiles, to validate genome annotations, to quantitate samples and their differential expression patterns, to map proteins in cellular compartments, and much more. Over all, proteomics is a functional genomics strategy to study all protein components and functions of a system, i. e. a cell, a tissue or an organism.

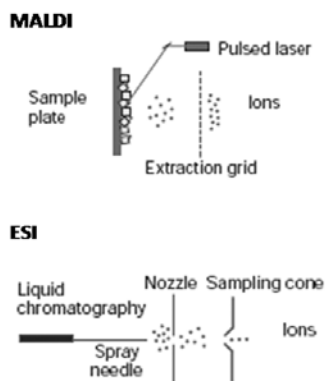
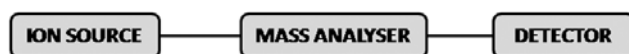
### 8.1 MASS SPECTROMETRY BASED PROTEOMICS

Mass spectrometry, a technique to measure the mass to charge ratio ( $m/z$ ) of a particle, dates back to the 19<sup>th</sup> century. But it was not until the late-1980s that an implementation for the study of proteins was first used. The development of two ionization methods: matrix assisted laser desorption ionization (MALDI) (Karas and Hillenkamp 1988) and electrospray ionization (ESI) (Fenn, Mann et al. 1989), made it possible to transfer a population of proteins or peptides into the gas phase atmosphere of a mass spectrometer, in a stable state.

MALDI requires the sample to be mixed with an organic matrix that absorbs light from a laser. Irradiation of the matrix-sample complex by the laser causes peptides desorption and ionization. This is followed by acceleration into the mass spectrometer (Westermeier 2002).

ESI produces ions by pressurizing the liquid sample through a thin capillary tube or needle under an electric field, at atmospheric pressure. The potential difference created in the space between the capillary and the mass spectrometer generates a cone of nanometer-sized droplets that fly through the inlet of the spectrometer (Westermeier 2002).

The basic components of a mass spectrometer are: an ion source, a mass analyzer that measures the mass-to-charge ratio of the analytes, and a detector (Figure VIII). As mention above, the ionization process can be either MALDI (MALDI-MS) or ESI (ESI-MS).



**Figure VIII: A simplified scheme of a mass spectrometer.**

The components: ion source, mass analyser and detector.

Below the ionization and sample introduction process in MALDI and ESI.

Modified from (Aebersold and Mann 2003) with permissions.

The instrumentation and principles of each mass spectrometer are far beyond the scope of this thesis, but two complete reviews which cover this area are referenced here (Aebersold and Mann 2003; Domon and Aebersold 2006).

### 8.1.1 The Sample

Before mass spectrometry analysis, the proteins in the sample are enzymatically digested into peptides and the peptides are concentrated. However, proteomics normally deals with complex mixtures of thousands of proteins that will generate an even higher number of peptides. A way to reduce the complexity of the mixture previous to the mass spectrometry analysis, is to separate proteins by gel electrophoresis and/or liquid chromatography.

Two-dimensional gel electrophoresis (2DGE) separates proteins in two steps, by isoelectric focusing (pI) (first dimension) and conventional SDS-PAGE (second dimension). The result is a two dimensional gel with spots that represent a single or a few co-migrating proteins. Each spot can be treated as an individual sample and the downstream detection becomes simpler.

Hydrophobic or membrane proteins are normally poorly represented in 2DGE and low-abundance proteins are masked in the gel by co-migrating highly abundant ones. This problem can be overcome by liquid chromatography.

Liquid chromatography (LC) is normally coupled to the mass spectrometer. LC can use of one (LC-MS) or two capillary columns (2DLC-MS), to concentrate and/or fractionate peptides after digestion (McCormack, Schieltz et al. 1997).

Both methods complement each other, and various combination strategies of one and two dimensional gel electrophoresis and liquid-chromatography mass spectrometry are also used to pre-fractionate the samples and simplify the subsequent detection.

These separations together with single mass spectrometry (MS) or tandem mass spectrometry (MS/MS), combined with different types of

instrumentation (mass analysers and detectors) have made mass spectrometry a highly sensitive strategy for the detection and characterization of proteins present even as only traces in the sample.

### 8.1.2 Result interpretation and data-handling

Results obtained from MS are analysed by specialized software. Data from MS are in the format of peaks in a spectrum. Each peak represents a peptide and a list of mass values for each peptide is generated. Each value carries associated statistical parameters, such as accuracy of the mass measurement, to support the results. The latest mass spectrometers have higher mass accuracy with greater specificity (Westermeier 2002; Domon and Aebersold 2006).

There are various software platforms to carry this type of search, for example MASCOT ([www.matrix-science.com](http://www.matrix-science.com)) and MS-FIT ([www.prospector.ucsf.edu](http://www.prospector.ucsf.edu)). They can be used online with limited features or be acquired for local use.

Peptide identification is the result of a search where the MS or MS/MS spectra are compared with calculated peptide mass after *in silico* digestion of the primary sequence protein database, derived from a DNA database. Without DNA databases (genomic or cDNA) available for the search, large-scale protein mass spectrometry would not be possible.

A precise match is retrieved if the protein is present in the database. When this is not the case, databases from other species can be searched and the software will pull out the closest homologous match.

## 8.2 ORGANELLAR PROTEOMICS

Mass spectrometry-based identification of proteins is also applied to the proteomes of subcellular structures and organelles. Pre-fractionation strategies are used to enrich samples for certain groups of proteins, and by doing so, increase the number of specific protein identifications compared to, for example, whole cellular proteomics (Taylor, Fahy et al. 2003).

This approach contributes to the determination of the location of the detected proteins, and thus provides functional data for the proteome.

However, there are a few points to consider for this approach. No structure or organellar fraction is normally 100% pure. Even if the identification of a protein in a “purified” organellar fraction is significant, it may not be part of the organelle in question. The sensitivity and accuracy of modern mass spectrometers are so high that is inevitable the detection of “contaminants” co-purified with the sample, even if using standard experimental controls, the fraction seems to be highly purified (Yates, Gilchrist et al. 2005; Andersen and Mann 2006).

Organelles are dynamic (Andersen, Lam et al. 2005). Proteins are constantly synthesized in one compartment and exported to the final location.



Transport proteins are constantly circulating via vesicles or components of the cytoskeleton. Cytoplasmic signaling proteins transiently associate to organelles and structures, via receptors or lipid anchors.

Integrative strategies must follow the mass spectrometry results, in order to assess the real location of the proteins identified. Among these strategies are: “subtractive proteomics”, which compares two sets of data, where one set has already been validated by other means (Andersen and Mann 2006); literature searches and bioinformatics tools, such as target signal predictions (pTARGET (Guda and Subramaniam 2005); Wolf pSORT (Horton, Park et al. 2007)) and sequence comparisons (BLAST (Altschul, Madden et al. 1997)); Gene Ontology (GO: [www.geneontology.org/](http://www.geneontology.org/)) where a conserved biological process, cellular component or function can be predicted (Au, Bell et al. 2007); fluorescent microscopy of fusion-tag expressed proteins detected by mass spectrometry (Warnock, Fahy et al. 2004; Gilchrist, Au et al. 2006); RNA interference (RNAi) and microscopy to detect the disappearance of a protein after gene silencing; among other downstream functional experiments.

## 9 THESIS RESEARCH

### 9.1 OBJECTIVES

In response to the large number of *T. cruzi* genes with unknown function coupled with the need for new specific drugs it is highly relevant to identify characteristics of trypanosomes that are essential for their survival, and that are different from those of the human host.

The general objective of the thesis was to identify and characterize novel proteins in *Trypanosoma cruzi*.

The aims were divided into two strategies:

1. Single gene identification and characterization, with the following particular aims:

- Detect novel polyprenyl synthases of the isoprenoid pathway, an essential process in parasite survival.
- Characterize the polyprenyl synthases at the biochemical and molecular level.
- Identify the locations of the polyprenyl synthases to determine if the isoprenoid pathway is compartmentalized in *T. cruzi*.

2. A large scale proteomics strategy with the following aims:

- Detect novel expressed proteins in *T. cruzi* epimastigotes.
- Identify organellar proteins by cell fractionation and mass spectrometry.
- Validate their cellular localization by microscopy, as a first step towards the determination of their function and biological importance.

### 9.2 RESULTS AND DISCUSSION

#### 9.2.1 Polyprenyl Diphosphate Synthases in *Trypanosoma cruzi*

As mentioned in the introduction (Chapters 4 & 5), polyprenyl synthases are important enzymes for the sterol metabolism and related pathways in the cell. I have here studied three enzymes from the isoprenoid biosynthetic pathway (Papers I to III). These are the only enzymes from this part of the pathway that have been described so far in *T. cruzi*.

#### **Paper I: A solanesyl-diphosphate synthase localizes in glycosomes of *Trypanosoma cruzi***

In Paper I, we reported the first characterization of a long-chain polyprenyl diphosphate synthase present in trypanosomatids, a solanesyl diphosphate synthase (TcSPPS).

The DNA sequence of *T. cruzi* solanesyl diphosphate synthase (TcSPPS) was extracted from a search through the EST (expressed sequence tag)

database of *T. cruzi* (Porcel, Tran et al. 2000) where it was annotated as a putative polyprenyl synthase.

*TcSPPS* is a 1092 bp single copy gene, as determined by southern blot analysis, and a restriction map analysis of the genomic contig (AAHK01002353.1) which held the complete gene sequence and both untranslated regions (5' and 3' UTRs). Both allelic copies are located in small chromosomes, 0.8 and 1.1 Mb.

*TcSPPS* is a protein of 363 amino acids (~39 kDa) that contains the seven conserved motifs of the polyprenyl synthase family, including the two aspartate rich motifs that are involved in catalysis and chain length determination. Analysis of the sequence surrounding the first aspartate rich motif (FARM), showed the presence of the small residue Alanine (Ala) at position -5. Amino acids at this position interfere with the extension of the isoprenoid product if they are bulky residues or allow the synthesis of a long chain isoprenoid if they are small amino acids. This was our first indication that this enzyme may be a long chain polyprenyl diphosphate synthase.

The recombinant *TcSPPS* was expressed in bacteria and tested for polyprenyl synthase activity. It was active and synthesized a 45 carbon isoprenoid or solanesyl diphosphate (SPP) as the maximum length product detected by thin layer chromatography. Solanesyl diphosphate is the substrate for synthesis of side chains of ubiquinone 9, the major ubiquinone synthesized in *T. cruzi*.

Involvement of *TcSPPS* in the ubiquinone synthesis was confirmed when the gene was able to complement a strain of *E. coli* deficient for the *ispB* gene involved in the synthesis of ubiquinone 8 in bacteria. *E. coli ispB* codes for an octaprenyl diphosphate synthase, a long chain polyprenyl synthase.

When catalytic conditions were assayed, the preferred substrate was GGPP as shown by a higher  $k_{cat}/K_m$  ratio. This higher affinity for GGPP is not common among long chain synthesizing enzymes.

Antibodies raised against the recombinant protein detected it in all three stages of *T. cruzi* (epimastigote, trypomastigote and amastigote) by western blot analysis, together with another band of 85 kDa. We hypothesized that the latter was probably an unknown protein or polyprenyl synthase that cross-reacted with the anti-*TcSPPS*.

The same antibody was used in immunofluorescence and immunogold electron microscopy. This revealed that the protein localized to the glycosome of *T. cruzi*. This was the first SPPS to be found in a peroxisome like organelle. Further confirmation was obtained from cell fractionation and differential centrifugation steps to enrich for glycosomes. Western blots over the fractionation samples, showed that the protein is glycosomal, although no glycosomal import signal was found in the sequence of *TcSPPS*. The 85 kDa protein was also detected, but only in the cytoplasmic and not in the glycosome enriched fraction.

Although several enzymes involved in the isoprenoid pathway have been described in peroxisomes, this is the first case for trypanosomes. The HMG-CoA reductase is the first step enzyme in the mevalonate pathway that precedes and continues into isoprenoid biosynthesis. It has been found to locate to the mitochondrion in trypanosomes. The IPP synthesized from

mevalonate is used by FPPS to produce FPP. TcFPPS has been described previously, but its location had not been assessed in *T. cruzi* or *T. brucei*.

This led me to determine the location of the FPPS, which is the subject of the next paper.

## **Paper II: Farnesyl diphosphate synthase localizes to the cytoplasm of *Trypanosoma cruzi* and *Trypanosoma brucei***

The farnesyl diphosphate synthases of *T. cruzi* and *T. brucei* were characterized previously (Montalvetti, Bailey et al. 2001; Montalvetti, Fernandez et al. 2003) and were shown to be targets of inhibition by biphosphonate derivatives, but its cellular location remained unknown. The fact that the FPPS from *Leishmania major* was shown to be cytoplasmic was not a good indication, since there are cases where the location of orthologue enzymes in kinetoplastids are different.

In this brief report, we showed that the farnesyl diphosphate synthase is indeed a cytoplasmic enzyme in *T. cruzi* and *T. brucei*, as shown by gradual cell permeabilization with digitonin and western blot analysis of the liberated soluble proteins in each step.

Immunofluorescence microscopy of colocalization assays with ER, mitochondrial and glycosomal markers showed that the TcFPPS and TbFPPS did not localize to these organelles.

When the rabbit anti-TcSPPS antibody was used in the blots of the extracts from the permeabilization with digitonin, the 85 kDa band was once more detected in the cytoplasmic fraction. We decided to begin the characterization of this protein.

## **Paper III: An unusual polyprenyl synthase from *Trypanosoma cruzi***

The work performed here is an initial characterization of a polyprenyl synthase from *Trypanosoma cruzi* that we named TcPPS, with unusual features compared to other members of this family of these enzymes.

The size of TcPPS is double that of the standard for PPSs due to the presence of a DUF2006 motif in the first portion of the protein. DUF2006 is a conserved domain of unknown function with no relation to polyprenyl synthase activity.

To confirm that the protein is actually this long, RT-PCR over total epimastigote RNA was performed and the size and sequence of the obtained transcripts confirmed the size and sequence of the annotated gene in the *T. cruzi* genome, which indeed codes for an 85 kDa polyprenyl synthase.

When TcPPS was expressed in bacteria, it was clear that the anti-TcSPPS crossreacts and also detects TcPPS in western blots. This showed that the protein is clearly localized to the cytoplasm of epimastigotes in *T. cruzi* as shown by the differential centrifugation steps in Paper I and the digitonin permeabilization assays performed here.

Analysis of the amino acid sequence, especially the region surrounding the first aspartate rich motif, showed that small residues are located at positions -5 and -4, which indicates that PPS is able to synthesize isoprenoids longer than FPP. We hypothesized that TcPPS could act as a GGPPS, as *T. cruzi* utilizes GGPP to prenylate proteins in the cytoplasm of epimastigotes.

The recombinant TcPPS was soluble and active in induced bacterial culture, and it appeared to be more active when DMAPP and GPP were the allylic substrates in the reaction mix. Although further characterization is needed, this preference for the smaller allylic substrates rather than FPP, as it is the case for other GGPPS, is another unusual feature of TcPPS, and complicates the functional picture.

## 9.2.2 Large scale proteomics in *T. cruzi*

### Paper IV: Proteomics in *Trypanosoma cruzi* – Localization of Novel Proteins to Various Organelles

In this paper, we have performed a mass spectrometry based proteomic study to detect novel proteins in organelles of the epimastigote stage of *T. cruzi*. Organelles from trypanosomes are particular to these parasites and they usually contain proteins with particular features, probably less abundant and probably masked under highly abundant cytoplasmic proteins. This study aimed to detect these proteins.

Differential and gradient ultracentrifugation steps after cell disruption allowed us to enrich for organelles from the parasite, and remove the majority of the highly abundant cytoplasmic and plasma membrane proteins.

The sample, resolved by 2DGE and 1D gradient gel electrophoresis, was subsequently subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS), to render around 400 protein identifications. The dataset included proteins annotated with a putative identity either because they were previously described or they show homology to known proteins in other species, and hypothetical proteins with unknown function.

By analyzing the results deeper, we discovered several isoforms of certain proteins, mainly in the 2DGE, and in some cases allelic versions with amino acid sequence differences. We could thus detect hypothetical proteins that were individually annotated as copies of the same gene product. The numbers of proteins in the different categories are shown in the table below:

|                                      | Total | Putative Identity | Hypothetical Proteins |
|--------------------------------------|-------|-------------------|-----------------------|
| <b>Identified proteins</b>           | 396   | 258               | 138                   |
| <b>2DGE</b>                          | 125   | 92                | 33                    |
| <b>1DGE</b>                          | 334   | 222               | 112                   |
| <b>Detected Protein Isoforms</b>     | 43    | 32                | 9                     |
| <b>Validation of expression data</b> | 173   |                   |                       |

The fractionation strategy allowed the detection and validation of the expression data of 173 proteins that had escaped detection by the whole cell proteome analysis published by Atwood *et al*, 2005.

A supplementary table was generated (Suppl. Table S7) where we presented results from searching through the bibliography databases for each of the 258 proteins detected in our sample with some identity to known proteins, in order to determine those where expression data was obtained by single gene characterization studies and to possibly locate them to cellular compartments. To our surprise, only for eight of the 173 novel proteins, we detected evidence from other reports of their expression in *T. cruzi* epimastigotes. The proteins were from several different organelles and some of them presented dual localizations. Almost 13% of the 258 protein set could not be assigned to any cell compartment.

To validate the organelle enrichment of our sample and begin to further characterize a number of hypothetical proteins, we cloned five genes into a specific *T. cruzi* expression vector, pTEX. The cloning produced a fusion tag to the epitope E910 from the c-myc gene. For this purpose we used the Gateway system that was not to our knowledge, previously tested in *T. cruzi*. Epimastigotes from the *T. cruzi* CL Brener strain, transfected with the generated vectors, were visualized after selection by immunofluorescence microscopy. All the five proteins were located in organelles (endoplasmic reticulum, mitochondrion, acidocalcisome, and cytoplasmic vesicles).

It is clear that the proteomic approach coupled with cell fractionation is a useful strategy to detect novel proteins, to obtain expression data, to validate genome annotations, and to obtain initial data to design follow-up studies for further protein characterization.

By looking into the long lists generated from the mass spectrometry data searches, proteins linking metabolic pathways can be detected, proteins thought to be present can be identified, and much more.

### 9.2.3 Conclusions

#### Papers I to III

Three polyprenyl diphosphate synthases are expressed in *Trypanosoma cruzi*:

1. The short chain enzyme, FPPS was described previously and localized here to the cytoplasm.
2. The *trans*-long chain SPPS, was localized to the glycosomes and functionally characterized.
3. An as yet putative “short chain” polyprenyl synthase, colocalized with FPPS.

The localizations of these three polyprenyl synthases expanded the sterol metabolism into the glycosomes and cytoplasm in *T. cruzi*, and the characterization was partially extended to *T. brucei* as shown in Paper II.

The TcHMGR, localized in the mitochondrion, produces mevalonate that will in turn produce isopentenyl diphosphate (IPP). We now know that IPP is required in the cytosol and glycosome as substrates of the polyprenyl diphosphate syntases. This means that the isoprenoid pathway it is not compartmentalized and that a transport of intermediates between organelles occurs. Whether this transport happens by passive diffusion of the metabolites or there is a much more controlled transport system, it needs further investigations.

Given the fact that TcPPS colocalizes with TcFPPS, is it possible that they form an active complex? If so, is the DUF2006 in TcPPS a structural domain that allows this protein to interact with FPPS, other proteins or even membranes? These questions that have arisen from our findings necessitate further investigation and may lead to further drug target identifications.

As was the case for TcSPPS, the recombinant protein was shown to be inhibited by biphosphonates derivatives (Szajnman, Garci et al. 2007), which validated the importance of our findings.

#### Paper IV

Protein expression data is important to validate genome annotations. It is also an important indication of the needs of the cell for a specific protein to be present and functioning.

We have detected almost four hundred proteins, located in different cellular compartments and contributed to the proteome of *T. cruzi* epimastigotes by detecting around 170 proteins with no previous expression data.

By immunofluorescence microscopy, we localized a novel metal transporter to the acidocalcisomes, which presence was hypothesized for years from the metal content in the lumen of this organelle. A novel protein was localized to the mitochondrion and two more were found to be associated with the endoplasmic reticulum. A fifth protein was distributed along the cell cytoplasm, and we hypothesized its location to be cytoplasmic vesicles, due to the presence of 14 transmembrane hydrophobic regions.

These findings contribute to the future design of methods to further characterize these proteins and determine their cellular function in *Trypanosoma cruzi*, and probably in other trypanosomes.

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