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Chapter

Role of Proteomics in the Study of *Trypanosoma cruzi* Biology

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Abstract

Proteomics is the science that studies the proteome, which corresponds to the global expression of proteins at a given time under determined conditions. In the last 20 years, proteomics has emerged as a powerful tool that has allowed the study of proteins that are expressed in the cell under normal or altered conditions as well as post-translational modifications, such as phosphorylation, glycosidation, acetylation, and methylation, among others. In this chapter, we present the main contributions of proteomics to the knowledge of *Trypanosoma cruzi* biology. Proteomes of all *T. cruzi* life cycle stages, secretomes/exoproteomes, post-translational modifications such as phosphorylation or acetylation and immunomes, interactomes, and glycomes are described. The role of proteomics in the identification of new chemotherapeutic targets and potential vaccine candidates will also be discussed.

Keywords: *Trypanosoma cruzi*, proteomics, subcellular proteomics, post-translational modifications, drug resistance, virulence, secretome/exoproteome

1. Introduction

The flagellate protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease, an illness that affects 6–8 million people worldwide [1]. The parasite has a complex life cycle, which starts when the triatomines defecate in the vicinity of the bite, and in the infective stage, the metacyclic trypomastigote (MT) is carried into the wound by scraping and then penetrates across the skin and invades resident cells. Once in the cytosol, MTs transform into amastigotes. This proliferative stage multiply in the cytosol and, after various rounds of binary fission, transform into trypomastigotes, which are released from the cell and disseminated by the bloodstream invading different organs, spreading the parasitic infection [2].

From a biological and biochemical point of view, *T. cruzi* is a genetically heterogeneous species with a wide variety of strains distributed in different vectors and with a great diversity of mammalian hosts. This high genetic variability is related to the variability of virulence and clinical manifestations among strains [3]. Currently, *T. cruzi* is classified into seven discrete typing units (DTUs), TcI to TcVI [4], and more recently, a new one named Tcbat has been included [5].

2. The proteomics and mass spectrometry

Proteomics is a discipline focused on understanding protein expression in biological samples and the possible comprehension of the complex physiological and

pathological events using different methods to identify and characterize proteins. Thus, proteomics is the study of the proteome, which corresponds to the global patterns of proteins that determine cell expression under a determinate condition [6].

From 1997, when the first paper on the proteome was published [7], proteomics studies have become an invaluable tool not only for the study of the global cell expression of proteins (the proteome) but also for the dissection of specific processes in cell biology and biochemistry, such as the study of proteins that are expressed in organelles and post-translational modifications [8, 9]. Moreover, by combining proteomics analysis and bioinformatics tools, researchers can report much broader projections, including the identification of new chemotherapeutic targets, new vaccine candidates, new tumor markers with prognostic or diagnostic value [10], and the study of secretomes and immunonomes, among others [11]. Thus, a current PubMed search with the keyword "proteomic" delivers 96,688 published papers.

Initially, Edman sequencing was used, and this method was replaced by mass spectrometry (MS). MS allows the separation of different isotopes, which is possible due to the use of soft ionization procedures, such as matrix-assisted laser desorption/ionization (MALDI) [12] and the electrospray ionization method (ESI) [13]. In both cases, the analytes (peptides) are ionized from the solid phase (MALDI) or liquid phase (ESI) into the gas phase. Then, liquid chromatography (LC) systems can be directly interfaced to mass spectrophotometers. LC tandem mass spectrometry (LC-MS/MS) has been applied to proteomics to separate peptides, and MS/MS records the intact peptides (full MS) before one precursor ion is selected and fragmented. This fragmentation is always induced in a collision cell by nitrogen or argon. Then, fragments are recorded in an MS/MS spectrum, and this fragmentation pattern reveals a specific mass for each amino acid from the peptide [6]. In summary, the amino acids present in the peptide can be determined, as well as the peptide sequence, and finally, the protein containing this peptide is identified.

In kinetoplastids like *T. cruzi*, all protein-encoding genes are organized in large polycistronic transcription units that produce polycistronic precursor RNAs that are then processed to monocistronic mRNAs by the mechanism of trans-splicing [14]. Then, translation of specific mRNAs and/or the stability control regulate the gene expression [15]. Furthermore, post-translational modifications perform a key function in order to modulate the *T. cruzi* protein function. For these reasons, in recent years, proteomics has had a crucial role in evaluating protein expression levels in different *T. cruzi* stages or following specific treatments with drugs, inhibitors, or natural products, and thus, proteomics studies have been transformed in an invaluable tool to reveal adaptive changes in this protozoan [16].

3. The *T. cruzi* proteome

A proteomic analysis of the human pathogen *T. cruzi* using two-dimensional electrophoresis (2-DE) was initially reported by Paba et al. [17]. A *T. cruzi* comparative proteome analysis of different stages was performed. This study showed that few proteins displayed significant differential expression among the three developmental *T. cruzi* stages. Using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry peptide mass fingerprinting, the researchers identified 26 proteins. Among these, 19 different proteins were identified, mainly heat shock proteins (HSP; chaperones, HSP 60, HSP 70, and HSP 90), elongation factors, glycolytic pathway enzymes (enolase, pyruvate kinase, and 2,3 bisphosphoglycerate mutase), and structural proteins (KMP 11, tubulin, and

paraflagellar rod components). In addition, the same researchers reported a comparative proteome analysis of *T. cruzi* developmental stages. In this study, isotope-coded affinity tag technology (ICAT) associated with liquid chromatography-mass spectrometry peptide sequencing (LC-MS/MS) was used. High-confidence sequence information and expression levels for 41 *T. cruzi* polypeptides, including metabolic enzymes, paraflagellar rod components, tubulins, and heat shock proteins, were reported. Twenty-nine proteins displayed similar expression levels in trypomastigotes as well as amastigotes, nine proteins displayed higher levels in trypomastigotes than amastigotes, and three proteins showed higher expression in amastigotes than trypomastigotes [18].

In addition, Parodi-Talice et al. [16] described a standardized and reproducible protocol for analyzing *T. cruzi* proteins by 2-DE, which generated protein reference maps of this parasite. In this report, tubulins, HSP, and prostaglandin F2α synthase were three of the most abundant proteins reported in *T. cruzi*.

A proteomic analysis of all *T. cruzi* stages was performed. Peptidic sequences from 2784 proteins in 1168 protein groups from the annotated *T. cruzi* genome were reported in all parasite stages. Protein products were identified from >1000 genes annotated as "hypothetical" in the parasite genome, including integrants of the mucin-associated surface proteins (MASP). The four parasite stages appear to use distinct energy sources, including histidine for stages present in the insect vectors and fatty acids by intracellular amastigotes [19]. In contrast, a study that identified basic proteins reported different enzymes related to amino acid metabolism in epimastigotes, whereas trans-sialidases and protein paraflagellar rods were found specifically in trypomastigotes [20]. Another proteomics study of *T. cruzi* trypomastigotes identified 1448 nonredundant proteins, 14% of which corresponded to surface proteins, and the majority were anchored via glycophosphatidylinositol (GPI) and related to parasite virulence [21]. According to these authors, the high diversity of expressed proteins on the trypomastigote surface could have implications for both invasion and immune evasion strategies. A comparison of the cell surface proteomes from different *T. cruzi* stages revealed that some enzymes are stage-specific, although the majority were present in more than one stage. Bioinformatics analysis showed that most of the identified proteins were derived from the membrane and were involved in cell invasion, adhesion, cell signaling, and host immune response modulation [22]. However, the biological role of these proteins was not experimentally demonstrated.

The blood trypomastigote (BT) proteome has also been described [23]. In a shotgun analysis, 17,394 peptides were reported, corresponding to 7514 proteins of which 5901 corresponded to *T. cruzi*. Trans-sialidases (TS), bioenergetics-related enzymes, chaperones, and cytoskeletal proteins were found among the top scoring proteins. Gene ontology (GO) studies showed that all *T. cruzi* compartments were evaluated and the most part of proteins were engaged in metabolic processes and/or presented catalytic functions. A comparative study among BT and tissue-culture-derived trypomastigote (TCDT) or metacyclic trypomastigote (MT) proteomic profiles identified 2202 proteins uniquely reported in the BTs. These unique proteins were related to (a) surface proteins, (b) the nonclassical secretion pathway, (c) cytoskeletal dynamics, (d) the cell cycle and transcription, (e) proteolysis, (f) redox metabolism, (g) biosynthetic pathways, (h) bioenergetics, (i) protein folding, (j) cell signaling, (k) vesicular traffic, (l) DNA repair, and (m) cell death [23]. The Brunoro paper identified many proteins belonging to different metabolic parasite pathways and functions that were exclusive to BTs and provided a valuable data set for a better biological understanding of this parasite stage.

The *T. cruzi* cell surface is covered by a dense layer of GPI-anchored molecules. These molecules are involved in a variety of interactions between the parasite and

its vertebrate and invertebrate hosts. GPI-anchored rich fractions and other *T. cruzi* membrane proteins were obtained from epimastigotes (EPIs) and MTs and then submitted to two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D-LC-MS/MS). A total of 98 MT proteins and 280 EPI proteins were identified. Of those, approximately 65% (n = 245) had predicted lipid post-translational modification sites (i.e., GPI-anchor, myristoylation, or prenylation), signal-anchor sequences, or transmembrane domains that could explain their solubility in detergent solution. This report showed that MTs, but not EPIs, express a large repertoire of surface glycoproteins, such as GP90 and GP82, which are involved in host cell adhesion and invasion. Taken together, these results showed that *T. cruzi* displays stage-specific protein profiles that are related to the biology of each *T. cruzi* stage [24].

A map of soluble proteins from epimastigotes of *T. cruzi* CL Brener was generated using 2-DE combined with MS. The analysis showed that several proteins, such as tubulin and heat shock proteins, were found in multiple isoforms. The enzyme arginine kinase was also found. This enzyme is absent in mammalian cells and, according to the authors, could represent a potential new chemotherapeutic target [25].

Using conditions optimized for the 6–11 pH range, researchers compared the proteomes of TCDTs and extracellular amastigote-like parasites by MALDI-TOF/TOF. The alkaline 2-DE gels from TCDTs and extracellular amastigotes showed that proteins with a pI over 7.0 were very well depicted (= 65% of proteins detected). Furthermore, the differences in protein expression among the human-hosted *T. cruzi* stages were in agreement with former proteomics reports and were related to the biological properties of each parasite stage [26].

4. The *T. cruzi* secretome/exoproteome

Adhesion and invasion are the first stages of interaction between an obligate intracellular pathogen and its host cell. This process involves cell surface molecules and secreted molecules. Then, the molecules that are released from pathogens may play a critical role during the establishment of infection, especially in immunomodulation or immune evasion, as well as in migration across host tissues, cell adhesion, cell-cell communication, differentiation, proliferation, and morphogenesis [27, 28].

The term secretome was first introduced in a bioinformatics survey of proteins secreted by *Bacillus subtilis* [29]. The authors defined the secretome as the subset of the proteome that is secreted, in addition to the components of cellular machinery used for protein secretion. However, these released proteins are not only secreted proteins but also proteins that arise from other export mechanisms. Thus, the best term to describe the protein content found in the extracellular proximity of a given biological system is the "exoproteome" [30].

According to [31], *T. cruzi* releases proteins associated with two types of vesicles. The large vesicles (LVs), named ectosomes, arise from the plasma membrane, and small vesicles (SVs), similar to exosomes, are released by the flagellar pocket. A third fraction was enriched in soluble proteins and was not associated with extracellular vesicles (EVs) [31]. Label-free quantitative proteomics analysis showed the presence of proteins associated with metabolism, signaling, packaging of nucleic acids, parasite survival, and virulence [31].

The *T. cruzi* exoproteome of TCDTs (Y strain) was recently characterized. NanoLC-MS/MS analysis resulted in the identification of 540 proteins. Bioinformatics analysis predicted most identified proteins as secreted, predominantly by nonclassical pathways, and suggested that these proteins are involved in host cell interaction. Some proteins possess predicted GPI-anchor signals; these

proteins are mostly TS, MASP, and surface glycoproteins. The existence of various related proteins to similar functions in the exoproteome likely reflects this parasite's enhanced mechanisms for adhesion, invasion, and internalization of different host cell types and immune evasion [32].

Considering that Chagas disease is a chronic infection in which amastigote forms remain for a long time, the *T. cruzi* laboratory examination using direct observation is tricky, and although antibody detection tests are sensitive, they may give false-positive results. The implementation of novel fast assays aimed for the identification of excretory/secretory parasite-derived molecules in serum would be a useful contribution in the diagnosis of human *T. cruzi* infection. Brossas and coworkers [33] investigated the proteins secreted by *T. cruzi* using MS analyses of conditioned culture media devoid of serum, collected during the emergence of trypomastigotes from infected Vero cells. Indeed, the secretomes of two *T. cruzi* strains from DTU TcVI (VD and CL Brener) were compared. This study led to the identification of 591 *T. cruzi* proteins. Three hundred sixty-three proteins were common to both strains, and most belong to different multigenic super families (e.g., TcS, GP63, MASP, and DGF1). Finally, a report of 94 secreted proteins, present in both DTU TcVI strains that do not correspond to the members of multigene families, was also established. This paper offers the first comparative study of the secretomes from two different *T. cruzi* strains of DTU TcVI. Then, Brossas and coworkers' papers identified a subset of common secreted proteins that could have a potential role as serum biomarkers for *T. cruzi* infection [33].

A novel family of *T. cruzi* surface membrane proteins (TcSMPs), which are conserved among different *T. cruzi* lineages and have orthologs in other *Trypanosoma* species, was described. Several lines of evidence suggest that TcSMP is a membrane-spanning protein located at the cellular surface and is also released into the extracellular milieu. TcSMP displayed a signal anchor and a C-terminal hydrophobic sequence predicted to be a transmembrane domain. In addition, proteomic TcSMP peptides previously found in a membrane-enriched fraction were identified in membrane vesicles as well as in soluble forms in the *T. cruzi* secretome. TcSMP proteins were also located intracellularly, likely associated with membrane-bound structures. These proteins were shown to inhibit metacyclic trypomastigote entry into host cells. TcSMPs could bind to mammalian cells and trigger Ca^{2+} signaling and lysosome exocytosis. This evidence strongly suggests the involvement of these proteins in parasite invasion [34].

5. *T. cruzi* subcellular proteomics

A combination of MS-based proteomics methods and traditional biochemical cell fractionation protocols had been used to characterize subcellular organization. The protein contents of specific subcellular compartments can thus be identified following specific enrichment strategies that concentrate and enrich organelles and subcompartments of the cell [8].

T. cruzi reservosomes show concentrated proteins and lipids obtained from medium together with the main proteolytic enzymes originating from the secretory pathway, being at the same time a storage organelle and the main site of protein degradation and endpoint of the parasite endocytic pathway. By cell fractionation and LC-MS/MS, the EPI reservosome-resident proteins were identified in a total of 709 *T. cruzi*-specific proteins. Among these, 456 had predicted functions and 253 were described as hypothetical proteins. The existence of a high number of previously reported proteins was confirmed. In addition, new different classes of proteins were reported. Among them, transport proteins, proton pumps, and

enzymes were also identified. According to the authors, the definition of the reservosome protein profile could be a good tool to assess the molecular signatures, identify molecular markers, and understand the relationship with different parasite organelles [35].

The EPI transition from the exponential growth phase to the stationary phase is a key step that recapitulates the early molecular events of *T. cruzi* metacyclogenesis, opening new possibilities for understanding this process. Thus, a quantitative shotgun proteomics study of the *T. cruzi* EPIs in the exponential and stationary growth phases was performed. More than 3000 proteins were identified and quantified, stressing the regulation of proteins engaged in various subcellular compartments. Ribosomal proteins were positively regulated in the exponential phase, in agreement with the high proliferative rate of this growth phase. Autophagy-related proteins were positively regulated in the stationary growth phase, showing the beginning of the metacyclogenesis. Otherwise, this paper reported the regulation of N-terminally acetylated proteins during growth phase transitioning, adding a new layer of regulation to this process. Thus, this paper reported a proteome-wide rewiring during *T. cruzi* differentiation from the EPIs' proliferative exponential phase to the stationary growth phase, which is the step before cell transformation into the MT stage [36].

In the eukaryotic cell, chromatin-associated proteins are key regulators of many important processes. By high-resolution MS, the *T. cruzi* chromatin proteome was investigated using three different protocols and comparing it between two parasite stages, EPIs and TCDTs. More than 2000 proteins were identified and quantified both in chromatin and nonchromatin extracts. In addition to histones and other known nuclear proteins, trypanosome chromatin also contains metabolic proteins (mainly from carbohydrate pathways), cytoskeleton components, and many other proteins with unknown functions. In addition, EPIs and TCDTs also differ greatly regarding their chromatin-associated factor composition and amount. Although the nucleosome content was the same for both stages, the remaining proteins were rarely detected in TCDTs, suggesting that they have naked chromatin. Proteins associated with DNA proliferation, such as proliferation cell nuclear antigen (PCNA), replication protein A (RPA), and DNA topoisomerases, were exclusively found in the chromatin of EPIs. However, TCDTs showed an enrichment of a histone H2B variant. Moreover, nearly 20% of the EPIs' chromatin-associated proteins were expressed in TCDTs but were placed in the nonchromatin space. Several types of proteins, including phosphatases and a Ran-binding protein, that may be transported among chromatin and the nonchromatin space during differentiation were also identified. These results indicated that the replicative state in trypanosomes involves an increase in chromatin-associated protein content. Given that trypanosomes are early-branching organisms, these data could enhance our understanding of chromatin-associated processes in other cell types [37].

T. cruzi proliferation displays unique features, such as the absence of chromosome condensation and closed mitosis. Thus, *T. cruzi* epimastigotes were lysed, and the nuclear fraction was isolated to study the proteome by LC-MS/MS. A total of 864 proteins were identified, of which 272 were annotated as putative uncharacterized proteins and 275 had not been previously reported in the *T. cruzi* proteome. Bioinformatics analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was carried out, and proteins were grouped into nuclear proteins in 65 gene clusters, wherein the clusters with the highest enrichment scores harbored members with chromatin organization and DNA-binding functions [38].

Contractile vacuole complexes are critical components of cell volume regulation and have been shown to have other functional roles in several free-living protists.

However, very little was known about the functions on *T. cruzi*. A proteomics and bioinformatics approach to identify proteins localized to the contractile vacuole was performed using the *T. cruzi* epimastigote-enriched fraction of contractile vacuoles, which was analyzed by one-dimensional gel electrophoresis and LC-MS/MS. A total of 109 newly detected proteins were identified, including at least 39 members of the dispersed gene family 1 (DGF-1). This observation suggested that many members of this family are simultaneously expressed in EPIs. In addition, several homologs with known localizations in contractile vacuoles of other organisms were selected. GFP-fusion proteins or specific antibodies were used to determine the localization of each protein. Six of these putative proteins (Rab11, Rab32, AP180, ATPase subunit B, VAMP1, and phosphate transporter) were mostly located in the vacuole bladder. However, TcSNARE2.1, TcSNARE2.2, and calmodulin were located in the spongiome. Calmodulin was a cytoplasmic protein. These observations confirmed the usefulness of merged subcellular fractionation, proteomics assays, and bioinformatics methods in order to know the location of organellar proteins that could be hard to identify when global proteomic approaches are used [39].

Several lines of evidence suggest that the translational system in trypanosomatids shows important differences compared to those of other eukaryotes. A careful data mining quest for ribosomal protein genes in the *T. cruzi* genome database coupled with MS of isolated *T. cruzi* ribosomes was performed. The findings suggested that *T. cruzi* ribosomal proteins have around 50% sequence identity to yeast proteins. In addition, some trypanosomal proteins were longer than yeast proteins because of the existence of many N- or C-terminal extensions, which are unique to trypanosomatids. In detail, L19 and S21 have shown C-terminal extensions of 168 and 164 amino acids, respectively. Even more, two 60S subunit proteins that had not been formerly found in the *T. cruzi* whole proteome, namely, L22 and L42, were detected [40].

6. Parasite biology and reversible post-translational modifications

In eukaryotes, histones are well-conserved proteins that form the basic structure of chromatin and undergo several post-translational modifications, which are important for the control of transcription, replication, DNA damage repair, and chromosome condensation [41].

The processes by which *T. cruzi* acquires infectivity and survives in different hosts involve rapid adaptations to new environments and tight regulation of gene expression, mainly post-transcriptionally. Nevertheless, the chromatin structure/organization of trypanosomatids is similar to that of other eukaryotes, including histone variants and post-translational modifications. Evidence suggests that epigenetic mechanisms also play an important role in the parasite, indicating that new epigenetic targets can be used for chemotherapeutic intervention. From this perspective, MS-based large-scale proteomics has been used to identify *T. cruzi* post-transcriptional modifications in *T. cruzi* canonical and variant histones. A total of 13 distinct modification types were identified, including 9 newly described chemical modifications for trypanosomatids, which included novel and unusual modifications, such as alternative lysine acylations, serine/threonine acetylation, and N-terminal methylation. In the same way, two hundred conserved and unique post-transcriptional sites in the tails and globular regions of all canonical and variant histones were identified. These results provided the first comprehensive map of *T. cruzi* histone marks and provided new evidence that allowed us to gain a deeper understanding of the epigenetic mechanisms that operate in *T. cruzi*. This report could provide information to identify potential candidates for the development of new epigenetic drugs [42].

In trypanosomatids such as *T. cruzi,* the regulation of gene expression is mostly post-transcriptional. The stability of mRNA and the possibility to accede to polysomes are believed to be strongly regulated, enabling *T. cruzi* to accommodate to various environmental situations that occur through its life cycle. Post-transcriptional regulation needs the association among mRNAs and specific proteins to shaping the complexes named ribonucleoproteins (mRNP). The dynamic association among proteins and mRNAs was studied through poly(T) beads to purify and characterize proteins and protein complexes linked to poly-A+ mRNAs. The protein contents of these fractions were studied by LC-MS/MS. A number of 542 protein components of the mRNP complexes associated with mRNAs were detected. Among them, 24 of the retrieved proteins were common to all fractions, whereas others were only found in an individual fraction: EPI polysomal (0.37%) and post-polysomal (2.95%) fractions and stress polysomal (13.8%) and post-polysomal (40.78%) fractions. This proteomic approach provided the first insight into the composition and dynamics of *T. cruzi* mRNPs [43].

Protein acetylation is also a post-translational modification that regulates several cellular pathways. Using proteomics, researchers identified 389 ε-lysine-acetylated sites in 235 proteins in *T. cruzi* epimastigotes. Lysine-acetylated protein sites were enriched in enzymes involved in oxidation/reduction balance, which is required for parasite survival in the host. These observations provide evidence that in *T. cruzi*, protein acetylation participates in the differential regulation of several cellular pathways and helps elucidate the mechanisms involved in parasite infection and survival [44].

In early-branched organisms, histones appear to be less conserved and contain alternative sites for modifications, which could reveal evolutionary unique functions of histone modifications in gene expression and other chromatin-based processes. Using high-resolution MS, researchers studied the *T. cruzi* trypomastigote and epimastigote histone post-translational modifications. A total of 44 new modifications were detected: 18 acetylations, 7 monomethylations, 7 dimethylations, 7 trimethylations, and 4 phosphorylations. Epimastigotes contained more histone modifications than trypomastigotes. Acetylations of lysines at the C-terminus of histone H2A and methylations of lysine 23 of histone H3 were reported to be enriched in MT or TCDT. Contrasting with that, protein phosphorylation at serine 23 of H2B and methylation of lysine 76 of histone H3 predominated in epimastigotes. In parasites undergoing mitosis and cytokinesis, the existence of one or two methylations in lysine 76 was reported. These observations helped reveal the role of histone modifications in gene expression control and cell cycle regulation in *T. cruzi* [45]. However, epigenetic events, such as histone acetylation, affected DNA topology, replication, and gene expression. Histone deacetylases are involved in chromatin compaction and post-translational modifications of cytoplasmic proteins, such as tubulin. Given these facts, histone deacetylase inhibitors, such as trichostatin A, were evaluated by de Oliveira Santos and coworkers to determine their effects on *T. cruzi* epimastigote cell proliferation, viability, cell cycle, and ultrastructure, as well as on histone acetylation and tubulin expression. A quantitative proteomics study showed an increase in histone acetylation after trichostatin A treatment, suggesting that deacetylase inhibitors may represent excellent tools for elucidating trypanosomatid cell biology [46].

Protein N-myristoylation is catalyzed by N-myristoyltransferase (NMT), an essential and potential drug target in *T. cruzi*. Roberts and Fairlamb used a combination of label-free and stable isotope labeling of cells in culture (SILAC)-based proteomics approaches when parasites were treated with or without the N-myristoyltransferase inhibitor DDD85646. A total of 56 proteins present in at least two out of the three experimental studies were identified. Among these,

6 were probably to be false positives, with the remainder 50 starting with the amino acids MG at the N-terminus in one or more of the *T. cruzi* genomes. The greater part of these were proteins of unknown function (32), with the remaining (18) involved in a different spectrum of key cellular and metabolic roles, such as intracellular transport, cell signaling, and protein turnover. In conclusion, the results showed that 0.43–0.46% of the *T. cruzi* proteome corresponded to N-myristoylated proteins, slightly lower than that reported in other eukaryotic organisms (0.5–1.7%) [47].

Glycoproteins are very attractive therapeutic candidate targets because they mediate key processes during the *T. cruzi* life cycle, such as cellular recognition, host cell invasion and adhesion, and immune evasion. Using subcellular fractionation, lectin affinity, and SILAC, Atwood et al. [48] conducted a *T. cruzi* trypomastigote glycoproteomics analysis, which described the identification of organelles and cell surface N-linked glycoproteins of *T. cruzi*. These researchers identified 36 glycosylation sites on 35 glycopeptides that mapped to 29 glycoproteins. They also presented the first evidence for 11 *T. cruzi*-specific glycoproteins and provided experimental data indicating that the MASP and dispersed gene family (DGF-1) were post-translationally modified by N-linked glycans. In another study using lectin-based and hydrophilic interaction liquid chromatography followed by high-resolution LC-MS/MS, a comprehensive glycoproteomics analysis was performed in *T. cruzi* epimastigotes and trypomastigotes. After treatment with glycanases, a number of 1306 N-glycosylation sites in NxS/T/C motifs were reported from 690 *T. cruzi* glycosylated proteins. Among them, 170 and 334 glycoproteins were solely detected in EPI and trypomastigotes, respectively. In addition, global site-specific characterization of the N- and O-linked glycan heterogeneity in the two *T. cruzi* life stages was reached by intact glycopeptide methods, detecting 144/466 unique N-linked and 10/97 unique O-linked intact glycopeptides in epimastigotes/trypomastigotes, respectively. Thus, *T. cruzi* displays a pattern of stage-specific glycoprotein expression that may be the result of parasite interactions with vertebrate and invertebrate hosts during its life cycle [49].

In eukaryotic cells, SUMOylation is an important protein post-translational modification. The C-terminus of proteolytically activated small ubiquitin-like modifier (SUMO) is covalently bound to a lysine residue of the target protein by an isopeptide bond through a pathway that involves an E1-activating enzyme, an E2-conjugating enzyme, and a transfer to the target, in occasions with the support of a protein ligase [50]. The modification is reversed by a protease, which is also responsible for SUMO ripening. Several proteins have been described as SUMO targets and were shown to participate in the regulation of cell cycle progression, transcription, translation, ubiquitination, and DNA repair [50]. Orthologous genes corresponding to the SUMOylation pathway have been reported in *T. cruzi*. Furthermore, the SUMOylation system appears to be functionally active in the parasite. An immunofluorescence analysis showed that *T. cruzi* SUMO (TcSUMO) was predominantly found in the nucleus. To identify SUMOylation targets and further elucidate their physiological roles, researchers generated transfectant *T. cruzi* epimastigote lines expressing a double-tagged *T. cruzi* SUMO, and SUMOylated proteins were enriched by tandem affinity chromatography. By two-dimensional LC-MS/MS, a total of 236 proteins with different biological roles were reported as possible *T. cruzi* SUMO targets. Among these, metacaspase-3 was confirmed as a bona fide SUMOylation substrate by a biochemical approaches. The application of proteomics methods in many different biological systems has allowed to know that orthologs of putative *T. cruzi* SUMOylated proteins are equally modified, suggesting that conserved mechanisms could operate for protein SUMOylation [51].

For the role of phosphorylation, kinase inhibitors have been suggested as novel antiparasitic agents. However, a fundamental understanding of the cell signaling pathways requires a detailed analysis of the involved phosphorylated proteins. A MS-based phosphoproteomic map of *T. cruzi* epimastigotes was performed by LC-MS/MS, dual-stage fragmentation, and multistage activation method. A total of 237 phosphorylated peptides from 119 different proteins were identified. Moreover, 220 phosphorylation sites were unequivocally mapped: 148 on serine, 57 on threonine, and 8 on tyrosine. Even more, immunoprecipitation and immunoblot assays reported the existence of at least seven tyrosine-phosphorylated proteins in *T. cruzi*. The phosphorylated proteins were submitted to GO, InterPro, and BLAST scan and classified according to their roles in cell structure, motility, cellular transport, metabolic pathways, pathogenesis, DNA/RNA/protein turnover, and signaling. These phosphoproteomic information provided novel perspectives into *T. cruzi* phosphorylation pathways and the potential roles of the identified phosphoproteins in *T. cruzi* biology and reinforced the idea that kinases are parasite drug targets [52].

Furthermore, a phosphoproteomic approach was performed to determine the role of transforming growth factor beta (TGF-β) in *T. cruzi* biology. Then, EPI (Y strain) was incubated with TGF-β for 1, 5, 30, and 60 min, which promoted remodeling of the parasite phosphorylation network and protein expression pattern. To identify parasite proteins involved in the TGF-β response, researchers performed 2-DE and MS. The modified proteins were engaged in many parasite functions, such as proteolysis, metabolism, heat shock response, cytoskeleton arrangement, oxidative stress regulation, translation, and signal transduction. After TGF-β treatment, a number of 75 protein spots were found to be positively or negatively regulated more than twofold. Among these, 42 were detected by MS, highlighting, among these, cruzipain, the main *T. cruzi* papain-like cysteine protease. In these experiments, TGF-β addition favored epimastigote proliferation, corroborating 2-DE data in which proteins previously described to be involved in this process were positively stimulated by TGF-β [53].

The MT proteome, which showed differential expression of some proteins during metacyclogenesis [54], has also been determined. Metacyclogenesis is the process by which *T. cruzi* epimastigotes differentiate into metacyclic trypomastigotes and acquire the ability to infect cells and resist complement lysis. This process obviously requires the expression of a new set of proteins associated with parasite virulence. This critical step in parasite biology was first studied by Parodi-Talice and coworkers [55] who analyzed the process by 2-DE coupled to MALDI-TOF MS. A large proportion of unique proteins expressed during metacyclogenesis were observed. Interestingly, 50% of the spots were found to differ between epimastigotes and trypomastigotes. The study provided a 2D map of MT. Sixty-six protein spots were successfully identified, corresponding to 43 different proteins. The expression profiles allowed protein identification during metacyclogenesis and categorize the proteins into three groups in accordance to their maximal level of expression. Various isoforms for a number of proteins, some of them displaying differential expression during metacyclogenesis, were detected. These information suggested that post-translational modifications may be an essential component of the parasite's biological program to regulate gene expression during metacyclogenesis. The Parodi-Talice and coworkers' paper contributed to the identification of relevant proteins implicated in the metacyclogenesis process, providing relevant information about the pathways involved in *T. cruzi* epimastigote differentiation into MTs [55]. However, a quantitative MS-based proteomics study of *T. cruzi* metacyclogenesis was performed, and almost 3000 proteins expressed during the process were quantified. Relevant proteins and pathways involved in the parasite's differentiation and infectivity

acquisition were identified, opening new perspectives for further studies that could lead to the identification of new chemotherapeutics targets [54].

Studies of the *T. cruzi* phosphoproteome and the nuclear subproteome were also performed [56], and the results showed that the *T. cruzi* nuclear subproteome was composed of several members of the HSP family and members of the "retrotransposon hot spot" (RHS) protein family as reported by De Castro Moreira Dos Santos et al. (2015).

The differentiation from trypomastigotes into amastigotes, called amastigogenesis, has also been studied *in vitro* by incubating TCDTs in acidic DMEM to perform a comprehensive quantitative proteomics and phosphoproteomics analysis of *T. cruzi* amastigogenesis. Regulated proteins and pathways involved in coordinating amastigogenesis were also identified, indicating that a significant proportion of the regulated proteins were membrane proteins. The authors further reported that modulated phosphorylation events coordinated by protein kinases and phosphatases are involved in signaling pathways and were triggers after incubation in acidic medium [22].

One of the least studied differentiation processes is the passage of infective trypomastigotes to epimastigotes, termed epimastigogenesis [57]. To resolve this issue, researchers established an *in vitro* and *in vivo* model for epimastigogenesis, enabling the characterization of a new infective morphological stage called "recently differentiated epimastigotes." This stage was infective and resistant to complement-mediated lysis both *in vitro* and *in vivo*. Shotgun proteomics of all *T. cruzi* stages revealed that several proteins were upregulated only in recently differentiated epimastigotes (including ERO1, multidrug resistance protein E and ABC transporters). This evidence suggests a role for these proteins in complement-system resistance, as previously observed for other organisms [58, 59]. As indicated by the proteomics analysis, this stage contains a minimal and distinct set of molecules required for *in vitro* and *in vivo* infectivity, suggesting a putative role of epimastigotes in *T. cruzi* infection of mammalian cells [60].

A validated, constraint-based model of the core metabolism of *T. cruzi* strain CL Brener was carried out. The model includes 4 compartments (extracellular space, cytosol, mitochondrion, glycosome), 51 transport reactions, and 93 metabolic reactions covering carbohydrates, amino acids, and energy metabolism. In addition, several replicate high-throughput proteomic data sets were used to specifically examine the metabolism of *T. cruzi* epimastigotes. This paper shows the usefulness of constraint-based models for incorporating several sources of information (e.g., genomics, primary biochemical literature, proteomics) to build testable hypotheses. This model constitutes and focuses on the systematic analysis of *T. cruzi* metabolism under a broad spectrum of conditions and disturbances and should possibly contribute to identify new drug targets [61].

T. cruzi is always subjected to sudden temperature shifts during its life cycle. The adaptive capacity to these changes is critical for parasite survival, reproduction, and transmission. These changes may modify the profile of protein expression that participates in homeostasis during the course of stress treatment. Thus, the proteome of *T. cruzi* EPI exposed to heat shock was analyzed by 2-DE followed by MS for protein identification. A total number of 24 spots differing in abundance were reported. Of the 24 modified spots, 19 showed a higher intensity and 5 showed a smaller intensity in relation to the control. Various functional categories of the reported proteins were determined: metabolism, cell defense, hypothetical proteins, protein fate, protein synthesis, cellular transport, and cell cycle. Proteins engaged in the interaction with the cellular environment were reported too [62].

A proteomics analysis of an organellar cell fraction from *T. cruzi* CL Brener epimastigotes was reported by Ferella et al. [63]. A total number of 396 proteins were

determined by LC-MS/MS. Among these, 138 were reported as hypothetical in the genome databases, and the remainder was allocated to many metabolic and biosynthetic pathways, transport, and structural functions. Comparative studies with an entire cell proteome are made possible to validate the expression of 173 additional proteins. Among these, 38 proteins previously detected in other stages were not found in the only large-scale study of the total EPI proteome. A chosen set of identified proteins was further studied in order to know their gene copy number, sequence variation, transmembrane domains, and targeting signals. The genes were cloned, and the recombinant proteins were expressed with a c-myc epitope tag in *T. cruzi* EPIs. Immunofluorescence microscopy showed the location of these proteins in different cellular compartments, such as the ER, acidocalcisome, mitochondrion, and putative cytoplasmic transport or delivery vesicles. These pieces of evidences reinforce the idea that the use of enriched subcellular fractions allowed the identification of *T. cruzi* proteins that were unnoticed by whole cell proteomics approaches [63].

7. Proteomics in experimental chemotherapy and drug resistance

Chagas disease chemotherapy is based mainly on benznidazole (Bz) and nifurtimox (Nx), developed more than four decades ago, which are very efficient nitroderivatives against the acute stage but present limited efficacy during the chronic phase. In fact, both are far from ideal due to substantial secondary side effects, limited efficacy against different parasite isolates, the need for long-term therapy, and their well-known poor activity in the late chronic phase. Thus, identification of improved drugs to treat *T. cruzi*-infected people is urgently needed. Even though many different kinds of natural and synthetic compounds have been described to have *in vitro* and *in vivo* activity against *T. cruzi*, since the introduction of Bz and Nf, only scarce chemotherapeutic compounds, such as allopurinol and some sterol inhibitors, have moved to clinical assays.

The proteome of two *T. cruzi* strains after piplartine treatment was reported [64]. Considerable changes in the expression of enzymes involved in parasite protection against oxidative stress, such as tryparedoxin peroxidase (TXNPx) and methionine sulfoxide reductase (MSR), were observed in both strains. These findings suggested that inhibition of these enzymes could be potential chemotherapeutic targets.

The trypanocidal effects of naturally occurring quinones and their derivatives and naphthoimidazoles derived from β-lapachone N1, N2, and N3 were the strongest. To assess the molecular mechanisms of action of these compounds, researchers studied treated bloodstream trypomastigotes by 2D-DIGE followed by MALDI-TOF/TOF. A total of 61 differentially abundant protein spots were identified when comparing the control with the N1, N2, or N3 treatment for 34 identified spots. The differentially abundant proteins included activated protein kinase C receptor, tubulin isoforms, asparagine synthetase, arginine kinase, elongation factor 2, enolase, guanine deaminase, heat shock proteins, hypothetical proteins, paraflagellar rod components, RAB GDP dissociation inhibitor, succinyl-CoA ligase, ATP synthase subunit B, and methionine sulfoxide reductase. These information allowed the identification of different modes of action for N1, N2, and N3, which indicate a great variety of metabolic pathways involved and open new avenues for the development of new trypanocidal drugs [65].

In addition, proteomics was used to identify proteins involved in N1, N2, and N3 trypanocidal activity. In epimastigotes, naphthoimidazoles are involved in multiple mechanisms: (a) redox metabolism, (b) energy production, (c) ergosterol biosynthesis, (d) cytoskeleton assembly, (e) protein metabolism and biosynthesis, and

(f) chaperone modulation. These compounds were able to induce an imbalance in crucial pathways of the parasite, leading to the loss of metabolic homeostasis and *T. cruzi* death [66].

A proteomic analysis of *T. cruzi* resistance to Bz was reported initially by Andrade et al. [67]. The differential proteomes of *T. cruzi* with selected *in vivo* resistance to Bz (BZR and Clone27R), its susceptible pairs (BZS and Clone9S), and a pair from a population with benznidazole *in vitro*-induced resistance (17LER) and the susceptible pair 17WTS were studied by 2-DE followed by MS. Among 137 spots studied through MS, 110 were detected as 56 distinct proteins. Among the 56 different proteins, 36 were detected in resistant, 9 in susceptible, and 11 in both phenotypes. In the group of the proteins identified in resistant samples, five were found in Cl 27R and in BZR (calpain-like cysteine peptidase, hypothetical protein conserved 26 kDa, putative peptidase, peroxiredoxin, and tyrosine amino transferase) and four in Cl 27R and 17LER (cyclophilin A, glutamate dehydrogenase, iron superoxide dismutase, and nucleoside diphosphate kinase). However, the proteins detected in Bz-susceptible samples, PGF-2a, were found in BZS and 17WTS. A functional category study has shown that proteins engaged in transcription and protein destination were overexpressed in the Bz-resistant phenotype. This report provided large-scale, proteomic information to obtain a better knowledge of the mechanism involved in *T. cruzi* resistance to Bz [67].

8. Laboratory diagnostic and protein identification

Immune complexes (ICs) are the direct result of humoral immune responses. Therefore, the identification of foreign constituents or autoantigens within ICs might provide new insights into the immunopathology of infectious diseases. Ohyama and coworkers [68] studied the immune complexome of plasma samples obtained from *T. cruzi*-infected patients. Then, 20 seropositive plasma samples, including cardiac and/or megacolon chronic patients (n = 11) and asymptomatic patients (n = 9), were analyzed together with 10 seronegative healthy people to investigate the antigens linked to circulating ICs. In this study, 39 *T. cruzi* antigens and 114 human autoantigens particular to patients with Chagas disease were identified. In this report, two *T. cruzi* antigens (surface protease GP63 and glucose-6-isomerase) and six human autoantigens (CD180 antigen, ceruloplasmin, fibrinogen beta chain, fibrinogen beta chain isoform 2 preprotein, isoform gamma-A of fibrinogen γ-chain, serum paraoxonase) were reported in more than 50% of the patients evaluated. Human isoforms short of complement factor H-related protein 2 and *T. cruzi* TS were most often found in the asymptomatic forms of Chagas disease (5/9 for both) compared with the determinate Chagas (0/11, P = 0·046 for human, 1/11, P = 0·0498 for *T. cruzi*). The authors conclude that the Chagas disease immune complexome may reflect the difference in immune status among different clinical stages of chronic form of *T. cruzi* infection [68].

In a successful translation of proteomic-based studies into accessible tools for bench diagnosis, Ruiz-Lancheros and coworkers [69] reported the value of apolipoprotein A-1 and fibronectin fragments as markers of parasitological cure for congenital Chagas disease in Bz-treated children [69].

Using a proteomics approach, researchers identified a *T. cruzi* protein phosphatase 2A in a cytosolic extract previously purified in a microcystin-Sepharose affinity column. This strategy enables the cloning and expression of this protein phosphatase, which is involved in *T. cruzi* differentiation from trypomastigotes to amastigotes [70]. A proteomics approach was also used to identify a *T. cruzi* alfa 5 proteasome subunit, previously recognized by the monoclonal antibody 7E5 [71].

9. Host-parasite interaction

To investigate changes in the cells' phosphoprotein pattern after infection, researchers used proteome profiler human phospho-kinase arrays to investigate the molecular mechanism of *T. cruzi*-induced alteration of the colon during the early infection phase of primary human colonic epithelial cells (HCoEpiCs) infected with *T. cruzi* trypomastigotes at different time points. Significant changes in the phosphorylation pattern that could be related to cellular deregulation in colonic epithelial cells after parasite infection were observed, including a significant increase in the levels of phosphorylated heat shock protein (p-HSP) 27 and transcription factors such as c-Jun and CREB. A significant upregulation of phospho (p-) Akt S473 and p-JNK, which may directly or indirectly modulate CREB and c-Jun phosphorylation, respectively, was also observed together with increased levels of phosphorylated CREB and c-Jun in the nucleus. In addition, p-c-Jun and p-CREB colocalized in the nucleus after 180 min of *T. cruzi* infection. A greater level of p-c-Jun and p-CREB has been associated with inflammatory and profibrotic responses. *T. cruzi* infection of HCoEpiCs provokes an enhanced expression of thrombospondin-1 (TSP-1), which is fibrogenic at higher levels. The authors also found that *T. cruzi* infection influenced the expression of some transcription factors, such as NF-kB and JAK2-STAT1, which can enhance proinflammatory flux. Bioinformatics studies of the phosphoprotein networks derived using the phosphoprotein information serve as a blueprint for *T. cruzi*-mediated cellular differentiation of HCoEpiCs in the lapse of the acute phase of parasite infection.

T. cruzi was shown to release vesicles containing a wide range of surface molecules to affect the host immune responses and cell invasion [72]. A proteomics study reported different compositions of extracellular vesicles released by two *T. cruzi* strains associated with their distinct interaction with host cells [73]. The secretomes of two distinct *T. cruzi* strains (Y and YuYu), which were previously shown to differentially modulate host immune responses, were compared. Extracellular vesicles (EVs) obtained from tissue culture-derived trypomastigotes of both strains were purified and quantitated. Proteomics studies confirmed the higher concentrations of proteins encoded by the trans-sialidase (TS) gene superfamily, MASPs, and several well-known exosomal proteins in the YuYu strain, which also have shown a significant difference among purified EVs and the vesicle-free fraction compared to the Y strain. In order to know if those differences were associated with *T. cruzi* infectivity, J774 phagocytic cells and LLC-MK2 non-phagocytic cells were previously incubated with isolated EVs from both strains and then infected with TCDTs from Y strain. EVs liberated by the YuYu strain provoked smaller levels of infection but an increased intracellular proliferation in J774 macrophages than EVs from the Y strain. On the other hand, YuYu strain-derived EVs induced greater levels of infection of LLC-MK2 cells than Y strain-derived EVs. In summary, the quantitative and qualitative differences in EVs and released proteins from distinct *T. cruzi* strains may be correlated with the parasite capacity to be infective and virulent [73].

The involvement of host cell-derived microvesicles (MVs) during the early interaction between *T. cruzi* MTs and THP-1 cells was previously demonstrated. To determine the contribution of different parasite stages and their extracellular vesicles in the interaction with host cells, researchers assessed TCDTs, MTs, and EPIs, which were shown to provoke distinct levels of MV liberated from THP-1 cells. Nevertheless, only MTs and TCDTs could enhance parasite cell invasion. Fluorescence resonance energy transfer microscopy showed that THP-1-derived MVs can fuse with parasite-derived MVs. Additionally, MVs released from the TCT-THP-1 interaction displayed a bigger fusogenic ability than those from the META- or EPI-THP-1 interaction. However, by proteomics, a greater concentration

of proteins from MTs (25%) than TCDTs (12%) or EPIs (5%) was detected in MVs from the parasite-THP-1 interaction. Lastly, sera from patients with chronic Chagas disease at the indeterminate or cardiac phase were able to identify antigens in THP-1-derived MVs resulting only from interaction with META or TCDTs forms. Elucidation of the cellular traffic pathways and the role of MVs in modulation of host immune system could supply significant hints with respect to Chagas disease pathophysiology [74].

T. cruzi has two tryparedoxins (TcTXNI and TcTXNI) pertaining to the thioredoxin superfamily. TXNs are oxidoreductases that have a role in electron transfer between trypanothione and peroxiredoxins [75, 76]. This represents a difference with the mammalian cells, in which these roles are performed by thioredoxins. These differences make TXNs a new and interesting chemotherapeutic target. *T. cruzi* tryparedoxin 1 (TcTXN1) is a cytoplasmic and constitutively expressed protein in *T. cruzi*. To elucidate the *T. cruzi* redox interactome, an active-site mutant protein lacking the resolving cysteine was designed. Then, the *in vitro* complex formation among the mutated TcTXN1 and its natural partner, the cytoplasmic peroxiredoxin, was validated. By the expression of this mutant protein in parasites with an additional 6xHis-tag, heterodisulfide complexes were purified and identified by 2-DE/MS. This allowed us to identify 15 TcTXN1 proteins that were involved in 2 main processes: oxidative metabolism and protein synthesis and degradation. This type of experiment allowed the discovery of several putative TcTXN1-interacting proteins and contributed to a better understanding of the redox *T. cruzi* interactome [77]. In another work, the study was extended to TcTXNII. TcTXNII is a transmembrane protein anchored to the mitochondria surface and endoplasmic reticulum, with a cytoplasmic orientation of the redox domain. This protein is expressed during the metacyclogenesis process. To further characterize the redox interactome of *T. cruzi*, an active-site mutant TcTXNII lacking the resolving cysteine was designed, and through the expression of this mutant protein and incubation with *T. cruzi* proteins, heterodisulfide complexes were isolated and identified by mass spectrometry. This approach allowed the identification of 16 TcTXNII-interacting proteins that are involved in a wide range of cellular processes. Thus, this report indicated the relevance of TcTXNII and contributed to a better understanding of the *T. cruzi* redox interactome [77].

TcI isolates of *T. cruzi* exploit the antioxidant network for enhanced intracellular survival in macrophages and virulence in mice. In these reports, the TcI strains Colombiana (COL) and SylvioX10/4 (SYL) and a cultured clone (TCC) displayed distinct biological features in an experimental murine infection, including high parasitemia and symptomatic cardiomyopathy (SYL), low parasitemia and high tissue tropism (COL), and no pathogenicity (TCC). A proteomic study of the EPI and TCDT stages by 2-DE and MALDI-TOF MS, followed by functional annotation of the differential proteome data sets (≥twofold change, $P < 0.05$) demonstrated that (i) many proteins were engaged in cytoskeletal assembly and remodeling essential for flagellar wave frequency and amplitude and forward motility of the parasite and (ii) the parasite-specific antioxidant network was improved in COL and SYL (versus TCC) trypomastigotes. Immunoblots reveal the increased protein levels of cytoplasmic and mitochondrial tryparedoxin peroxidases and their substrate (tryparedoxin) and iron superoxide dismutase in COL and SYL (versus TCC) trypomastigotes. In addition, COL and SYL (but not TCC) were resistant to experimental treatment with oxidants such as H_2O_2 and peroxynitrite [ONOO(−)]. These parasite isolates were able to resist the intracellular superoxide and nitric oxide response in macrophages and were not killed by them. These observations indicate that protein expression focused on increased motility and control of macrophage-derived free radicals may represent a survival and persistence strategy developed by TcI isolates of *T. cruzi* [78].

10. *T. cruzi* typing

T. cruzi strains have been allocated to seven genetic groups (TcI-TcVI and TcBat), called discrete typing units (DTUs), which constitute groups of parasite isolates that differ in geographical origins, virulence, pathogenicity, and immunological properties [79]. In addition, several clinical manifestations (from asymptomatic to extremely severe illness) have been intended to be linked to *T. cruzi* genetic variability. For this reason, many DTU typing approaches have been proposed. Recently, a *T. cruzi* strain typing assay using MS2 peptide spectral libraries (Tc-STAMS2) was developed [80]. The Tc-STAMS2 methodology used shotgun proteomics associated with a spectral library quest to assign and discriminate *T. cruzi* strains independent of genome information. This approach was based on the built of a library of MS/MS peptide spectra constructed using genotyped *T. cruzi* reference strains. For identification, the MS/MS peptide spectra of unheard *T. cruzi* isolated were identified by the spectral matching algorithm SpectraST. The Tc-STAMS2 approach enabled right identification of all DTUs with high reliance. The approach was robust toward distinct sample preparations, length of chromatographic gradients, and fragmentation techniques.

The Tc-STAMS2 procedure allowed *T. cruzi* strain typing using MS/MS spectra as discriminatory functions and allowed the differentiation of TcI-TcVI DTUs. Similar to genomic-based procedures, the Tc-STAMS2 method permits identification of strains within DTUs. Its robustness toward different experimental and biological variables makes it an invaluable complementary methodology to the existing *T. cruzi* genotyping assays. Furthermore, this approach may be applied to identify DTU-specific properties correlated with the strain phenotype [73].

A proteomics analysis of two *T. cruzi* zymodeme 3 strains was also performed. In fact, two *T. cruzi* Z3 strains, designated 3663 and 4167, isolated from Brazilian Amazon triatomines were studied. These strains exhibited different infection patterns for Vero cells, in which 3663 trypomastigotes were much less infective than 4167 trypomastigotes. By proteomics, the differences in the global protein expression profiles of these two Z3 strains were investigated. Two-dimensional (2D) protein maps were obtained, and some spots were identified by MS. The study showed a meaningful difference in the expression profiles of several proteins in both strains. One of them was cruzipain, a key virulence factor. These data were corroborated by flow cytometric analysis using an anti-cruzipain antibody. This difference could contribute to the infectivity profiles observed for each strain by *in vitro* assays using different cell lines [81].

A phylogenetic character mapping of 26 stocks of *T. cruzi* and 2 stocks of the sister taxon *T. cruzi marinkellei* was performed to test for possible associations between *T. cruzi*-subspecific phylogenetic diversity and levels of protein expression, as examined by proteomics analysis and MS. A strong correlation ($P < 10(-4)$) was observed between genetic distance, as established by multilocus enzyme electrophoresis, and proteomic dissimilarities estimated by proteomic Euclidian distances. Several proteins were found to be specifically associated with *T. cruzi* DTUs. This study explored the previously uncharacterized links between *T. cruzi* infraspecific phylogenetic diversity and gene expression [82].

11. Immune response

One major problem in developing a rational search for *T. cruzi* vaccine candidates is the restricted information with respect to the proteins that are expressed by different strains, clones, and isolates included in the different parasite DTUs.

A proteomics study of *T. cruzi* trypomastigotes identified 1448 nonredundant proteins, of which close to 14% of the found peptidic sequences corresponded to surface proteins, mostly of which were GPI-anchored and associated with *T. cruzi* virulence. Immunoinformatics approaches reported a high number of peptides with predicted high-binding capacity for class I and class II molecules of the major histocompatibility complex (MHC). The many different types of proteins exposed on the trypomastigote surface membrane could have several consequences for parasite cell invasion and evasion of the immune response. Finally, a rational survey to identify potential T-cell epitopes that may be evaluated and validated to develop a prophylactic vaccine to protect against *T. cruzi* infection was performed [21].

T. cruzi BT proteins were immunoprecipitated using serum antibodies obtained from asymptomatic or cardiac (stages B1 and C) patients with different forms of the disease and from healthy people used as controls. Proteins recognized by sera from Chagas disease patients were identified and quantified by MS, and changes in the recognition patterns were further evaluated. Compared to asymptomatic samples, IgG from stage C patients mainly identify the I/6 autoantigen, whereas IgG from B1 patients resulted in a greater recognition of dihydrolipoamide acetyltransferase precursor, calpain cysteine peptidase, and two variants of CAP5.5. In this paper, CAP5.5 identification by human serum immunoglobulin from patients with early myocarditis resulted in a 23-fold abundance variation when compared to sera obtained from asymptomatic patients. These results strongly suggest the role of CAP5.5 in Chagas disease cardiomyopathy.

The lack of appropriate biomarkers to assess treatments and evolution of this disease is the main limitation for clinical trials and patient medical evaluation. Accordingly, these observations may contribute to a better comprehension of *T. cruzi* pathogenesis and to evaluating potential candidates for developing vaccine and diagnostic tests, in addition to the clinical applicability of suitable biomarkers for patient follow-up and prognosis [83].

The major protein antigens targeted by the diverse antibodies of *T. cruzi*-infected mice were studied. To detect global IgG antibody specificities, sera from infected mice were immunoblotted against whole *T. cruzi* extracts. The most immunogenic *T. cruzi* proteins were identified by proteomics using MALDI-TOF-TOF. Among these proteins, pyruvate phosphate dikinase, Hsp-85, and β-tubulin were recognized by mouse IgG as the major protein bands. The *T. cruzi* β-tubulin gene was cloned and expressed in *Escherichia coli*, and recombinant *T. cruzi* β-tubulin was used to immunize mice. Immunized animals increased their specific IgG reactivity and were protected against *T. cruzi* infection. These results strongly suggest that proteomics, used for repertoire analysis, is a valid tool to identify protective antigens that could be vaccine candidates to protect against *T. cruzi* infection [84].

12. Concluding remarks and future directions

T. cruzi infection is a major health issue in America and also in the rest of the world, where around 8 million people are infected by *T. cruzi* and the other 28 million are exposed to the parasite infection. Even more, ~20,000 people die each year, mainly as a result of cardiac problem. Currently, immunoprophylactic strategies have not been developed, and chemotherapy is so far from optimal, based on two old drugs, Nf and Bz. However, these drugs are more active in the acute form of Chagas disease and display several side effects.

In the last 15 years, a considerable number of proteomics studies have contributed to a better understanding of the biology and biochemistry of *T. cruzi*. In fact, the proteomes of the different life cycle stages of the parasite have been described,

including the proteomes of different zymodemes and different parasite DTUs. Using a typical experimental workflow as described in **Figure 1**, many different proteomics strategies have been reported. These allowed to determine the composition of different organelles, such as ribosomes, contractile vacuoles, reservosomes, and nuclear protein contents. These proteomics analyses have contributed to the knowledge of the products excreted/secreted by the parasite and have also allowed us to identify the contents of the microvesicles released by different parasite stages and their interaction with host cells. The study of post-translational modifications has also been possible and has contributed to the knowledge of parasite phosphorylation, glycosidation, myristoylation, SUMOylation, histone acetylation, and methylation. Proteomics has also helped elucidate critical biological processes, such as metacyclogenesis, amastigogenesis, and epimastigogenesis. The pathways involved in resistance to Bz and in the action mode of napthoimidazoles isolated from β-lapachone have been studied by proteomics approaches. Interactomics studies that demonstrated typing using M2 peptide spectral libraries and identification of cure markers are also among the major achievements of proteomics in the research field of *T. cruzi* and Chagas disease. In all of these cases, special care was taken in sample preparation, and it was always cleaned using "Stage tip" (or other close related system) in order to remove sample contaminants. In some experiments quantitative proteomics was performed using label-free methods, while in others labeling was performed using iTRAQ, ICAT, SILAC, or others. In all cases, digested proteins were submitted to liquid chromatography systems directly interfaced to mass spectrophotometers (**Figure 1**).

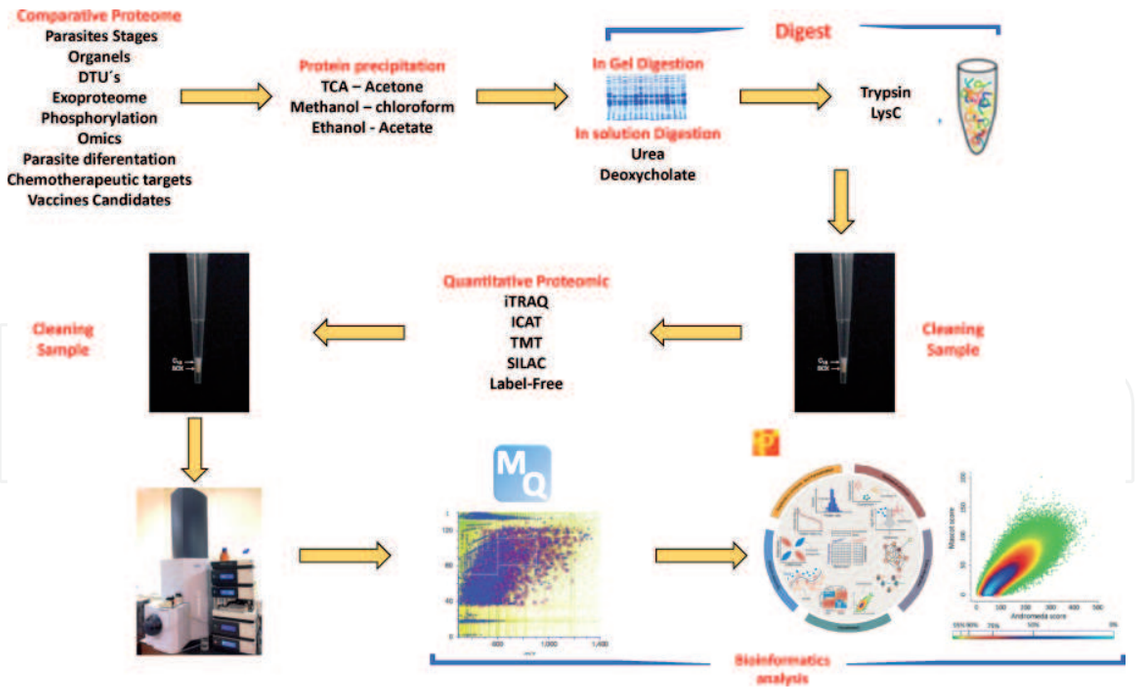

Figure 1.
Flow diagram of the main events in Trypanosoma cruzi proteomics. Qualitative and quantitative analyses using different parasite stages can be performed. Then, the proteins can be precipitated or separated by SDS-PAGE. Proteins are digested, in the gel or in solution using proteases, such as trypsin and Lys C. The peptides are subjected to a cleaning phase, and the peptides can be labeled using techniques such as ICAT, ITRAQ, and SILAC or not be labeled, using label-free quantification (LFQ). Finally, the samples are subjected to a ultrahigh-pressure liquid chromatography (UHPLC) associated to mass spectrometry (MS). The information obtained is submitted to bioinformatics analysis using software such as MaxQuant (or others), for quantitative proteomics analysis, and then by Andromeda to obtain the sequence of the peptides in the different databases and Perseus (or others) to obtain classifications, proteomics interactions, post-transductional modifications, and metabolic pathways.

However, we still expect major contributions of proteomics in many aspects of parasite biology and in various clinical aspects. The subproteomes of acidocalcisomes and the kinetoplast-mitochondrion complex could reveal important information about parasite biology.

In addition, the identification of new biomarkers with diagnostic and prognostic value and for established cure criteria is needed, and proteomics would contribute to this field. In the same way, the identification and validation of new chemotherapeutic targets to develop new and more efficient drugs and the identification of new potential vaccine candidates could also be an expected contribution.

Furthermore, in the future, considering the concept of "precision medicine" or "personalized medicine," proteomics will offer the opportunity to characterize strains isolated from infected patients, identify its DTU, and define appropriate treatments and perhaps appropriate immunoprophylaxis. Moreover, the identification of human sera biomarkers that allow us to predict which patients with the undetermined forms of Chagas disease show cardiomyopathy or digestive tract involvement would be a relevant contribution of clinical proteomics.

Recently, San Francisco et al. [85] reported that the *in vitro* invasion capacity and *in vivo* Balb/c mouse infectiveness of a highly virulent *T. cruzi* cell line were strongly reduced by parasite pretreatment with antisense oligonucleotides targeting trans-sialidase or complement regulatory proteins or with E64d to inhibit cruzipain activity. However, the parasite infectivity was not completely blocked, suggesting that other players, different from these proteins, could be involved in *T. cruzi* virulence. Thus, for comparison of the proteomic profiles of highly virulent and low-virulent cell lines of *T. cruzi*, the molecular bases of *T. cruzi* virulence will be established. For the *T. cruzi* secretome, different interactions among *T. cruzi* secreted proteins and host cell signaling pathways have been proposed [86]. However, the role of these proteins in virulence and modulation of the host immune system has not been shown experimentally. *T. cruzi* secretome studies and immunomics analyses using acute sera of animals or infected patients in the acute phase of the infection would help elucidate the mechanisms that *T. cruzi* uses to subvert the host immune response.

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